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The COXEN Principle: Translating signatures of *in vitro* chemosensitivity into tools for clinical outcome prediction and drug discovery in cancer

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Abstract

Substantial effort has been devoted to *in vitro* testing of candidate chemotherapeutic agents. In particular, the U.S. National Cancer Institute Developmental Therapeutics Program (NCI-DTP) Human Tumor Cell Line Screen has screened hundreds of thousands of compounds and extracts, for which data on more than forty thousand compounds tested on a panel of sixty cancer cell lines (NCI-60) is publically available. In tandem, gene expression profiling has brought about a sea change in our understanding of cancer biology, allowing discovery of biomarkers or signatures able to characterize, classify, and prognosticate clinical behavior of human tumors. Recent studies have used tumor profiling matched to clinical trial outcome data to derive gene expression models predicting therapeutic outcomes, though such efforts are costly, time-consuming, tumor type-specific, and not amenable to rare diseases. Furthermore, addition to new or established drugs to multidrug combinations where such models are already available requires the entire model to be re-derived. Can the aforementioned *in vitro* testing platform, coupled to the universal language of genomics, be used to develop, *a priori*, gene expression models predictive of clinical outcomes? Recent advances, including the COXEN (CO-eXpression ExtrapolatioN) algorithm suggests that this may be so and raise important implications for future trial design and drug discovery.

Introduction

Few technological innovations have transformed biology to the extent that has high throughput molecular profiling. Such efforts, coupled with a wide variety of platforms for statistical and biological interpretation as well as inter-institutional sharing of the data produced by these assays, have changed how we study neoplasia. Recent advances suggest that these technologies may change the way pathologists and oncologists approach the diagnosis and nosology of

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tumors, while key recent reports suggest that use of prediction frameworks developed from gene expression profiling data, roughly, “gene expression models (GEMs),” can be developed and clinically implemented to provide accurate prognostication of disease course (1) and even therapeutic benefit (2). For example, in bladder cancer, studies have developed GEMs predicting chemotherapeutic response of muscle invasive tumors (3,4).

While these efforts lay the foundation for implementation of molecular tools to predict patient benefit from standard cancer chemotherapeutic regimens, they come with important limitations. Such GEMs must be developed *a posteriori*, based on profiling patient tumors at baseline followed by supervised biomarker discovery comparing clinical responders and non-responders, with subsequent validation in independent trials. Thus, such strategies are not adaptable to rare orphan tumors (insufficient numbers for trials) or cancers of unknown primary (among the ten most common cancer diagnoses (5)). Such strategies also do not suggest a “salvage” therapeutic option for predicted non-responders, and such approaches are not adaptable for *a priori* stratification of patients for novel agents where clinical trial outcome is not known. Finally, upon addition of approved or investigational agents to standard combination regimens, existing GEMs must perforce be re-built and prospectively revalidated.

The United States National Cancer Institute's Developmental Therapeutics Program's (NCI-DTP) NCI-60 Human Tumor Cell Line Screen which has tested sixty cancer cell lines derived from nine common histologies tested with >110K compounds of which >45,000 are publically available, provides a rich database of *in vitro* drug response data (6). Initially intended as a government-sponsored drug discovery pipeline, this initiative has already made significant contributions directly to this objective. In addition, this data is a rich source of information that could be mined for additional biological insights. For example, reports as early as 2001 could demonstrate that using gene expression profiling of these sixty cell lines, coupled to the vast *in vitro* response data in the NCI-60 screen, investigators could develop signatures predictive of sensitivity within the same cell line panel (7). Taken a step further, what if the language of gene expression could be used to systematically extrapolate drug sensitivity results observed in cell culture screening to predict tumor behavior in patients? Surprisingly, only lately has this been demonstrated by us (8,9) and by others (10).

Motivation for the Development of the Coxen Algorithm

Bladder cancer-derived cell lines were not included in the NCI-60 cell line panel. Our desire to develop chemotherapeutic response prediction models for this tumor type prompted us to develop a collection of nearly forty commonly used bladder cancer cell lines, which we called BLA-40. These were profiled for their baseline gene expression using oligonucleotide microarrays, and tested *in vitro* for sensitivity to several chemotherapeutic drugs relevant in the treatment of urothelial cancer, including gemcitabine, cisplatin, and paclitaxel. Using a classification algorithm that favors discovery of robust, parsimonious gene expression models and is relatively resistant to “overfitting” (11), we were able to demonstrate in cross-validation studies correct prediction of drug sensitivity across the three drugs. Most compellingly, given the frequent use of doublet (gemcitabine/cisplatin) therapy for muscle invasive bladder cancer (12) we could predict response to doublet combination chemotherapy within the cell lines with 80% accuracy ($P=0.0002$) (13). We have recently reported a similar effort for the dual EGFR/HER2 inhibitor, lapatinib (14).

With this expression profiled bladder cancer cell panel in hand but lacking the resources to carry out large scale drug screening, we formulated the hypothesis that perhaps clustering of the NCI-60 gene expression data with that of BLA-40 would allow us to project the *in vitro* drug sensitivity data available on the NCI-60 to the bladder cancer cells lines. Unfortunately, this simplistic approach was not successful as the cell lines clustered primarily by histological

subtype. To correct for this, we first identified the genes whose expression in the NCI-60 was related to drug sensitivity and then determined which of these genes maintained *concordant expression* in the BLA-40 panel. This is performed through comparisons of correlation matrices. For example, for a list of 50 candidate sensitivity genes, a 50×50 matrix of the correlation of expression of the 50 genes, across the first cell line dataset, to each of the other 50 genes is generated. The same matrix is then prepared from the second cell line dataset gene expression data. Finally, each row (i.e., each gene/candidate biomarker) of these two correlation matrices is then correlated between the two matrices to generate a second correlation coefficient (the “COXEN coefficient”), reflective of whether that particular candidate biomarker maintains concordant relationships to the other candidate genes. A correlation coefficient cutoff may thus be used to select the concordant subset, which are then used for GEM development. Thus, this “correlation of correlations” step allows selection, through requiring intercohort concordant expression, of genes relevant to both cell line panels while maintaining strict independence of the datasets with respects to the drug sensitivity outcomes in the second cohort. We call this process COeXpression ExtrapolatioN, or COXEN, and have employed it for several combinations of cell line panel to cell line panel and cell line panel to human tumor projections as described below. COXEN was also used for computational drug discovery in bladder cancer using *in vitro* sensitivity data obtained from the NCI-60 panel. These applications are summarized in Figure 1.

Applications of Coxen in Therapeutic Response Prediction

Our first report of the development and use of this methodology described several applications of COXEN (8). The first was to use gene expression data for the NCI-60 cell lines, coupled with their published *in vitro* sensitivity data to the drugs cisplatin and paclitaxel, to predict sensitivity of our BLA-40 cell lines. By using a COXEN step to select concordant genes from NCI-60 data-derived candidate sensitivity biomarkers and implement them into GEMs, we were able to predict sensitive and resistant bladder cancer cell lines with accuracies of 85% for cisplatin and 78% for paclitaxel based on the NCI-60 cell line data alone.

The next application used gene expression and *in vitro* sensitivity data for paclitaxel and tamoxifen to predict actual therapeutic outcomes in two clinical studies where breast cancer patients had their tumors expression profiled. In the first case COXEN used paclitaxel *in vitro* cell line data to generate GEMs which were applied to the clinical trial response data for the related taxane, docetaxel. Three such models exhibited a classification accuracy of 75% for the 11 responders and 13 non-responders in this trial. A second clinical study-based implementation used a similar approach to predict metastatic recurrence of 60 estrogen receptor positive breast cancer patients treated with tamoxifen whose tumor tissues were gene expression profiled before therapy. Based on GEMs derived from concordant subsets of NCI-60 sensitivity data and untreated human breast tumors derived biomarkers, we were able to predict with 71% accuracy recurrence in this patient cohort (8).

Recently, we have undertaken an extensive validation of the COXEN methodology using data from an additional seven independent clinical trials of nearly 500 patients of diverse geographic and ethnic backgrounds (9). Importantly, 233 of these patients were from trials with prospective enrollment. We developed drug-specific GEMs, again, by comparing gene expression in cell lines from the NCI-60 screen based on *in vitro* sensitivity and used separate breast, bladder, and ovarian cancer gene expression datasets (without response data and unrelated to those trials examined in the study) for the COXEN step. Then, concordant sensitivity biomarkers were used to develop COXEN GEMs, which were evaluated singly and in combination for ability to predict response and survival outcomes for several published studies including bladder cancer patients (N=59) treated with methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), breast cancer patients (N=275) treated with 5-fluorouracil, doxorubicin, and

cyclophosphamide, and ovarian cancer patients (N=143) treated with combination platinum-based therapies.

Importantly, for two studies of bladder cancer tested, these COXEN GEMs performed well for patients in both the neoadjuvant (N=45) (3,15) and advanced tumor (N=14) (4) settings. When single drug GEMs for each of MVAC were combined, and prediction scores found to be significantly different between responders and non-responders ($P=0.002$ and 0.03 , respectively), resulting in a sensitivity of 83%, specificity 64%, positive predictive value of 71%, and negative predictive value of 78%. Compellingly, in the neoadjuvant dataset(3,15), which was large enough for multivariate analysis, the COXEN GEM scores were found to be independent of a variety of traditional clinicopathologic parameters and the only parameter independently associated with response.

For breast cancer and ovarian cancer, the COXEN GEMs developed from NCI-60 cell line testing performed similarly. While single drug GEMs for doxorubicin, cyclophosphamide, and 5-fluorouracil performed well in two separate studies gene expression profiled breast cancer drug outcome studies (N=133 and N=45, respectively), combination GEMs for these three drugs—used in both studies—were significantly associated with patient responses and survival status (as were the outcomes studied, respectively). This was associated with a sensitivity of 71% and specificity of 53%. In a third gene expression matched neoadjuvant study of gemcitabine, epirubicin, and docetaxel for breast cancer patients (N=100), each single drug COXEN GEM, as well as a three drug combination GEM, provided prediction scores significantly different between responders and non-responders. In first trial, the only case where complete clinical data were available for multivariate analyses, both ER status and the combination GEM were found to be significantly independently prognostic for response. Similarly, in two studies of ovarian cancer (adjuvant N=119, neoadjuvant N=24), COXEN GEMs for carboplatin and paclitaxel were both significantly predictive of response and survival, as well as independently predictive of response to therapy in the larger adjuvant trial where complete clinicopathologic data were available for multivariate analysis.

One interesting aspect of these findings that pertains to the inter-platform and inter-histology ability of COXEN was illustrated by a recent analysis we have subsequently performed on the breast and ovarian cancer studies from the above report. Wishing to determine whether cell line training set composition (as regards cell line tissue of origin) impacted the accuracy of prediction, we excluded breast cancer (N=5) or ovarian cancer (N=7) cell lines from the NCI-60 panel and re-derived GEMs. We found that the GEM predictions derived without breast or ovarian cancer cell lines included in training were essentially unchanged (*unpublished observations*). In the same vein, in the Williams *et al.* report, we did specifically examine whether the stage composition of the dataset used for the COXEN step (specifically, whether or not the tumors did or did not exhibit muscle invasion) changed the results. We found that either way it resulted in essentially identical outcome predictions (9).

Applications of Coxen in Computational Drug Screening

We have also reported an application of COXEN for *in silico* drug discovery. The publically available NCI-60 screening data for >43,000 candidate compounds, together with NCI-60 gene expression profiling, was used to develop drug-specific candidate biomarkers, concordant between the NCI-60 and BLA-40, which were implemented as GEMs predictive of sensitivity in the bladder cell lines *in vitro*. In fact, 139 compounds were identified for which >35% of the BLA-40 were predicted to be sensitive, of which eight had >50% predicted sensitive. The top hit for this screen was the cytotoxic imidazoacridinone, NSC-637993, which demonstrated empirically to exhibit sub-micromolar GI_{50} s in >60% of the BLA-40 cells. In comparison, the anchor drug of bladder cancer combination chemotherapy, cisplatin, exhibits submicromolar

GI₅₀s in only 22% (8). Perhaps more importantly, we also found that a structurally similar compound, C1311 was also a top COXEN “hit”. This finding is relevant because: 1) COXEN computational screening was able to find closely related analogs; 2) C1311 has significant cytotoxic activity *in vitro* and *in vivo* for a range of cancer cell lines (16); 3) C1311 has been tested in Phase 1 (17-19) and Phase 2 (20) trials for breast and other advanced solid tumors, potentially facilitating rapid translation of C1311 to bladder cancer patients.

In prior studies the cytotoxic activity of C1311 has been evaluated in 16 human cell lines from solid tumor types and leukemias producing a median IC₅₀ of 0.3 μ M (16). *In vivo*, C1311 caused 77% growth inhibition of HT29 colon cancer xenografts compared to 17% for paclitaxel (21). Importantly, no cross-resistance was seen between *in vitro* sensitivity to C1311 and doxorubicin or paclitaxel, two agents used in human bladder cancer, opening the possibility of effective combinations of C1311 with these agents. Consistent with these findings, we have recently completed testing of C1311 *in vitro* in our BLA-40 panel and have observed a median GI₅₀ of 0.5 μ M (Smith and Theodorescu, manuscript in preparation).

Questions and Future Directions

The ability of the COXEN methodology to produce, based solely on gene expression profiling coupled to *in vitro* NCI-60 testing, predictive models capable of stratifying patients is suggestive of several important applications that address limitations of gene expression models derived from a *posteriori* analysis of gene expression against patient treatment outcomes. Principally, these studies show that a *priori* stratification of patients is possible, offering the opportunity to substantially reduce the size of clinical trials for novel agents (22) while potentially avoiding treating patients who are unlikely to receive any clinical benefit from investigational or even standard of care regimens. For example, in the case of bladder cancer, cisplatin-based combination chemotherapy is associated with substantial morbidity and up to 3% mortality (12). The ability to stratify patients based on probability of response to standard therapies could spare the substantial proportion of therapeutic non-responders (~50% in larger studies)(12) the toxicity associated with therapy while preventing delay of surgery in the neoadjuvant setting (23). As authors have observed before, for advanced and inoperable patients, earlier triage of predicted non-responders to investigational agents could take place before further deterioration of performance status, a key factor for survival time in advanced disease (4).

In the case of investigational agents, particularly targeted agents, increasing experience in this area has suggested that a *priori* stratification of patients is essential to design trials enriched with responders and to exclude participants unlikely to enjoy any clinical benefit (22). Recent *a posteriori* studies have found that in some cases, particular genetic lesions confer exquisite sensitivity or resistance to targeted agents (24,25), while we note that, in the case of EGFR mutations and drug response in non-small cell lung cancer (NSCLC), between ~5-15% of mutation negative patient still enjoy a therapeutic response (24). We suggest that constructing GEMs *a priori* based on *in vitro* response by using COXEN might offer the opportunity to represent a core responder phenotype with gene expression that can be further refined when combined with targeted evaluations such as mutations or activity of signaling intermediates. Supporting this idea is a recent report by Balko *et al.*, who reported signatures of gefitinib sensitivity from *in vitro* studies that were predictive of EGFR mutation and activation status in NSCLC tissues(26). In addition, there is no reason that predictive nomograms could not be built based on traditional clinicopathologic characteristics, mutation or activation markers and yet include a GEM; indeed, we have incorporated phosphoprotein biomarkers into the development of combination GEMs for prediction of BLA-40 sensitivity to lapatinib (27).

This technology raises the spectre of other possible implementations. For example, future studies *in vitro* and in animal models might demonstrate that COXEN GEMs can predict efficacious agents for model “non-responders” to standard of care agents. Could such a strategy be implemented to suggest an approved agent or combination for rare “orphan” tumor histologies, where standard of care consists of only empiric multi-agent therapies because large randomized trials are not feasible? Most provocatively, could this type of technology be employed to offer other approved anticancer agents to predicted standard of care non-responders? Similar issues have been raised by other investigators in the field, who have questioned whether prospective validation might always be necessary, particularly in critical cases such as second or third line therapy for treatment failures where no standard of care exists (28). While the practical and ethical concerns of these kinds of implementations are manifest and deserve serious consideration and debate, it is clear that novel trial formats will be necessary to evaluate such interventions that are *eo ipso* outside of current designs. One potential way to address these concerns may be to use methodologies like COXEN GEMs to select therapies to add to standard of care regimens, as is often the trial design used to demonstrate efficacy for promising agents. Clearly, though, complex pharmacologic interactions will need to be considered in any combination that is computationally recommended.

From the standpoint of development, two significant opportunities are offered by COXEN. This technology can be used early on to evaluate which tumor histologies are most likely to respond to newly developed agents, suggesting ways to enrich early clinical trials with such patients increasing the chances for success. This approach could also be used in the unfortunate case of failed clinical trials for a “drug salvage/repositioning” strategy in another tumor system, with the theoretical ability to find success in a different tumor histology after trial failures in its primary target. Such trials would be especially efficient and more rapid since the pharmacology and toxicity of the drug would have already been well documented in prior studies.

Finally, while we have used COXEN to link cell line panels to other panels or to human tumors by virtue of the common language of gene expression, the principle is not limited by the platform. As such, we are in the process of evaluating the ability of COXEN to generate predictive biomarkers using DNA, micro RNA, as well as proteomic datasets from tumors and body fluids.

Caveats and Limitations

Several limitations must be taken into consideration and interpretation of the COXEN findings. First, save 233 of the breast cancer patients in the most recent report, the patient cohorts in which we have studied the predictive ability the COXEN GEMs were not prospectively enrolled, which can introduce selection biases. Though this limits the generality of interpretation of the potential role of GEMs in such a setting, it is reassuring to have observed significant prediction, retrospectively, in smaller patient subsets across multiple tumor types reported from geographically different areas. It is revealing, however, that recently this issue has come to the attention of the U.S. Food and Drug Administration, who have suggested in their non-binding recommendations, *Draft Guidance: In Vitro Diagnostic Multivariate Index Assays* (29), that “use of archived samples and/or retrospective data may sometimes be used to support clearance or approval, provided the study design and sample composition reflect the intended use of the device in the intended population.”

Beyond the larger concerns of how and under what circumstances COXEN or other powerful predictive technologies may be used or approved for use in patients (28), COXEN raises important technical implementation questions. For example, it certainly is not customary in

the general practice of oncology for most cancer types to subject patients to the additional morbidity of biopsy of, let alone metastectomy, for presumed metastatic disease. If COXEN GEMs are capable of predicting sensitivity in such a setting, is the original, primary tumor from the definitive resection even the correct lesion to profile for prediction? If not, what degree of efficacy, however defined, of predictive GEMs must be demonstrated to outweigh the risk of invasive surgical or imaging-guided sampling procedures (as has been reported recently (30)) to secure tissues of the metastasis itself to use for prediction. This kind of question becomes all the more important in the setting of metastatic recurrence post-adjuvant chemotherapy, as occurs with some frequency in bladder cancer, as such lesions are often chemoresistant and trials of second-line agents generally observe low response rates. Perhaps circulating tumor cells (CTCs) could be evaluated for the COXEN derived GEMs (31), and this may be a solution that obviates the need for biopsy in patients where such tumors can be captured and analyzed.

Conclusion

In summary, the COXEN algorithm shows promise as a means to use genomic information as a universal language to translate drug sensitivity biomarkers between cell line and tumor platforms. The findings reviewed herein constitute the first attempt at laying a foundation for future implementations that appear compelling and suggestive. Future studies ranging from model systems and preclinical studies to prospective clinical trials will determine if this concept, now in its infancy, will grow up into a strong adult in the fight against cancer.

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Figure 1. The COXEN algorithm and its implementations

Reported uses of the COXEN algorithm have included both intercohort prediction of sensitivity between different cell line panels for drug discovery and cell line to tumor prediction for response stratification in clinical trials. Either way, the workflow proceeds per the sequence illustrated at left. The algorithm starts by deriving candidate biomarkers by comparing gene expression data between sensitive and resistant cell lines from the NCI-60, a step which can be regarded as biomarker discovery. Then candidate biomarkers are triaged by COEXpression Extrapolation (COXEN) to ensure concordant expression between datasets. Then concordant biomarkers are used to derive gene expression models (GEMs), predictive of sensitivity to individual drugs or combinations. Last, such models used to classify cell lines or tumors based on their own gene expression data, and prediction scores examined with respects to empirical (in vitro) or observed (clinical trial) response outcomes. In the case of drug discovery, GEMs can be derived for each of thousands of drugs tested against the NCI-60 panel, then drugs ranked by percentage predicted responders to prioritize them for preclinical evaluation.