Phosphotyrosine Signaling Analysis in Human Tumors Is Confounded by Systemic Ischemia-Driven Artifacts and Intra-Specimen Heterogeneity

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Abstract

Tumor protein phosphorylation analysis may provide insight into intracellular signaling networks underlying tumor behavior, revealing diagnostic, prognostic or therapeutic information. Human tumors collected by The Cancer Genome Atlas program potentially offer the opportunity to characterize activated networks driving tumor progression, in parallel with the genetic and transcriptional landscape already documented for these tumors. However, a critical question is whether cellular signaling networks can be reliably analyzed in surgical specimens, where freezing delays and spatial sampling disparities may potentially obscure physiologic signaling. To quantify the extent of these effects, we analyzed the stability of phosphotyrosine (pTyr) sites in ovarian and colon tumors collected under conditions of controlled ischemia and in the context of defined intratumoral sampling. Cold-ischemia produced a rapid, unpredictable, and widespread impact on tumor pTyr networks within 5 minutes of resection, altering up to 50% of pTyr sites by more than 2-fold. Effects on adhesion and migration, inflammatory response, proliferation, and stress response pathways were recapitulated in both ovarian and colon tumors. In addition, sampling of spatially distinct colon tumor biopsies revealed pTyr differences as dramatic as those associated with ischemic times, despite uniform protein expression profiles. Moreover, intratumoral spatial heterogeneity and pTyr dynamic response to ischemia varied dramatically between tumors collected from different patients. Overall, these findings reveal unforeseen phosphorylation complexity, thereby increasing the difficulty of extracting physiologically relevant pTyr signaling networks from archived tissue specimens. In light of this data, prospective tumor pTyr analysis will require appropriate sampling and collection protocols to preserve in vivo signaling features. Cancer Res; 75(7); 1495–503. © 2015 AACR.

Introduction

Protein posttranslational modifications (PTM), such as phosphorylation, regulate the stability, localization, and activity of cellular components (1–3). Accordingly, dynamic phosphorylation plays a vital role in coordinating information flow within the cell and regulating emergent tumor responses ranging from proliferation to invasion and angiogenesis. Phosphorylation occurs predominantly on serine and threonine residues with tyrosine phosphorylation accounting for only approximately 0.05% of all phosphorylation events in eukaryotic cells (4). Although tyrosine phosphorylation is rare and tyrosine kinases represent only 0.3% of the genome, these enzymes represent close to 30% of the known oncoproteins such as SRC, EGFR, and BCR-ABL (5). Their disproportionate role in oncology, combined with their structural druggability, makes tyrosine kinases highly desirable therapeutic targets. Quantitative analysis of protein tyrosine phosphorylation in human tumor tissue specimens can provide insight into intracellular signaling networks underlying tumor behavior while identifying activated kinases and their substrates, signaling components that may represent druggable targets.

Human tumors collected by The Cancer Genome Atlas (TCGA) program potentially offer the opportunity to characterize activated signaling networks driving tumor progression, in parallel with the genetic and transcriptional landscape already documented for these tumors (6–8). The National Cancer Institute’s Clinical Proteomic Tumor Analysis Consortium (NCI-CPTAC) seeks to provide proteomic characterization of tumors genomically annotated by TCGA programs (9). State-
of-the-art mass spectrometry (MS)-based proteomics provides quantitative, systematic analysis of protein phosphorylation profiles (10–12) with the potential to directly identify activated signaling networks in tumors; information that is difficult to derive from genetic-based studies. However, tumor specimen analysis is complicated by possible temporal delays in tissue acquisition and processing along with spatial sampling differences due to the heterogeneous nature of human tumors. Freezing delays following sample resection and processing subject the specimen to ischemia; unfortunately the exact time to freezing for archived tissue specimens is often undocumented. To date, studies on the effects of ischemia on tissue phosphorylation have been reported, yet the sample collection and analysis approaches used did not allow assessment of short periods of ischemia (13) or permit comprehensive analysis of pTyr signaling (14).

The heterogeneous nature of human tumors has been widely documented (15, 16). Intercellular and intratumoral variations are evident at a macroscopic histologic level and at molecular, genetic, and epigenetic levels through clonal evolution and tumor microenvironment influences (17, 18). A previous report investigated phosphorylation patterns across distinct anatomic metastatic lesions of prostate cancer. However, the number of phosphorylation sites profiled was limited and intralesion comparison was not performed (19). The influence of proximal spatial heterogeneity within a tumor specimen on pTyr signaling networks has yet to be determined. Collectively, these preanalytical factors may influence protein phosphorylation measurements, thereby obscuring physiologic signaling networks (20). Therefore, we sought to investigate how preanalytical variations in sample collection and processing can ultimately affect downstream pTyr signaling analysis of human tumors.

Materials and Methods

Human ovarian and colon tumor collection

High-grade serous ovarian carcinoma tissue from 5 patients was collected as previously reported (21). Colon adenocarcinoma biopsy tissue was collected from five patients at the Cooperative Human Tissue Network at Vanderbilt University Medical Centre (CHTNI-VUMC; Nashville, TN), in accordance with Institutional Review Board–approved protocols. Following vessel ligation, surgical specimen removal was performed and the first core biopsy was taken immediately thereafter (t = 0), transferred to prechilled cryovials, and snap frozen in liquid nitrogen. Further core biopsies were collected and frozen after 10, 30, and 60 minutes of cold ischemia. Only specimens meeting pathology quality inclusion criteria of left-sided colon adenocarcinoma cancers in which clamp time could easily be determined, minimal tumor diameter of 4 cm, and no prior chemotherapy and/or radiation were released for analysis.

Protein extraction, digestion, and iTRAQ labeling of peptides from ovarian and colon tumors

Approximately 50 to 100 mg (total wet weight) of each of the timepoint samples was homogenized separately for protein extraction and digestion as described previously (22). Desalted peptides were labeled with multiplex iTRAQ (Isobaric Tags for Relative and Absolute Quantification) reagents as reported previously (22). Briefly, 800 μg peptide per sample for each of the ovarian and colon tumor timepoints was labeled with two tubes of iTRAQ reagent (according to the labeling scheme shown in Supplementary Figs. S2 and S5).

Phosphotyrosine peptide enrichment

Phosphotyrosine peptides were enriched before mass spectrometry analyses using a cocktail of anti-phosphotyrosine antibodies followed by immobilized metal affinity chromatography as previously described (22).

Mass-spectrometry–based phosphotyrosine analysis

Peptides were chromatographically separated and subsequently analyzed by Orbitrap Elite mass spectrometer (Thermo Scientific), database-searched, validated, and normalized as previously reported (22).

Phosphotyrosine data analysis

The total list of peptides and proteins identified and quantified can be found in Supplementary Tables S1, S2, and S4. All mass spectra, in the original instrument vendor format, contributing to this study may be downloaded from: https://cptac-data-portal.georgetown.edu/cptacPublic/

Mass-spectrometry–based protein expression analysis

Approximately 10% (~300 μg) of iTRAQ-labeled peptides from each colorectal tumor pTyr IP supernatant was separated off-line on C18 column. A total of 80 fractions were collected, noncontiguously pooled into 20 final fractions, and each subject to an independent LC/MS-MS analysis. Each fraction was separated by reverse phase UHPLC (Easy-nLC 1000, Thermo Scientific) before nanoelectrospray directly into a Q-Exactive mass spectrometer (Thermo Scientific).

Protein expression data analysis

Peptide and protein identification was performed with the Proteome Discoverer software (version 1.4; Thermo Scientific) using Mascot search engine (version 2.4.1, Matrix Science). MS-MS spectra were searched against a human protein sequence database (NCBI, 2012 release, 35,586 sequences). For each protein expression experiment, one combined database search was performed where the data files from all 20 independent MS fraction analyses were searched collectively as a single input dataset. The total list of peptides and proteins identified and quantified can be found in Supplementary Table S5.

Affinity propagation clustering analysis

Quantitative temporal profiles of all pTyr sites within an individual patient dataset were clustered using the affinity propagation algorithm proposed by Frey and Dueck (23). Individual patient datasets were subjected to independent analysis (see Supplementary Fig. S4) as described previously (22).

Statistical analysis and annotation

Hierarchical clustering analysis and heatmap construction were performed using the built-in Bioinformatics Toolbox function "Clustergram" in Matlab (R2013b, The Mathworks Inc.) with Euclidean pairwise distance metric. Statistical analysis was conducted using GraphPad Prism 5.0a software. Pearson correlation analysis (two-tailed), and pairwise Student t tests (two-tailed) were
used for calculating the significance of the differences and significance was accepted when $P < 0.05$. Kinase enrichment analysis was performed using the webtool at http://amp.pharm.mssm.edu/lib/kea.jsp and conducted as described previously (24). The input dataset was compiled from the union of ischemia-regulated pTyr sites found in >4 ovarian tumor samples and >4 colon tumor samples. Predicted kinases were plotted onto a dendrogram of the human kinome (25) using the webtool at http://web.ccs.pdx.edu/~josephl/kinome-cluster/. Panther Gene ontology (GO) annotations were identified by uploading UniProt ID lists to the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org/).

**Results**

Postexcision ischemia induces rapid, widespread, patient-dependent alterations to pTyr networks

Postresection ischemia time is generally undocumented in TCGA samples but may range from minutes to an hour during processing and pathologic inspection. To understand the effect of cold-ischemia on protein tyrosine phosphorylation, we collected patient-derived ovarian tumor samples from five individuals undergoing debulking surgery and performed a controlled ischemia 4-point time-courses (Supplementary Fig. S2). Ischemia time-courses from each patient were analyzed separately, providing detection and quantitation of several hundred pTyr sites per patient (Supplementary Table S1). Within 5 minutes of cold-ischemia, the fraction of pTyr sites within a specimen that showed quantitative fluctuations ranged from 29% to 55%, depending on the patient (Fig. 1A and Supplementary Table S1). Intriguingly, the temporal response to ischemia was patient specific. Two patients, 39 and 67, showed a predominant decrease across many pTyr sites within the first 5 minutes, whereas the other patients had a more mixed response, with comparable proportions of increasing and decreasing sites. We hypothesized that some of the inter-patient differences in response to ischemia could be attributed to patient-specific baseline phosphorylation profiles. To investigate this possibility, the "0 minute" samples from each ovarian patient were analyzed simultaneously by quantitative multiplex MS, allowing the relative levels of each pTyr site at resection to be directly compared between patients. Euclidean distance hierarchical clustering indicated variation in the basal pTyr network states at excision (Fig. 1B and Supplementary Table S2). This result agrees with previous reports noting that tumors with similar driver mutations can demonstrate distinct biologic interpretations if tumor specimens intended for pTyr analysis are not immediately frozen.

Spatial phosphorylation heterogeneity is apparent in human tumor samples

The heterogeneous nature of human tumors has been described (15–18). However, the effects of intertumor spatial heterogeneity on cellular signaling networks are unknown. To understand the impact of spatially resolved samples on protein phosphorylation profiles, we analyzed the pTyr profiles of paired spatially distinct primary colorectal tumor samples from five individuals undergoing resection (Supplementary Fig. S5 and Supplementary Table S4). As with the ovarian tumors, a controlled ischemia time-course enabled analysis of pTyr dynamics and the generality of ischemia effects across tumors. Unsupervised hierarchical clustering of intratumoral paired-timepoint samples indicated the presence of pTyr heterogeneity in spatially proximal samples (Fig. 2A), as indicated by the greater difference in the paired time-point samples (i.e., 0a and 0b) compared with the non-paired points (i.e., 0b and 10a in patient 041). In patients 323 and 745, where the 0 minute paired timepoints clustered together, significant differences were still apparent. Technical measurement error did not contribute significantly to the observed variation, and was not the cause of the observed heterogeneity (Supplementary Fig. S6). Pearson correlation analysis of the quantitative pTyr data for each pair of time points (Supplementary Fig. S7) indicated weak correlations and minimal shared covariance between spatially proximal samples, even in tumor 323, the most "homogeneous" tumor, where paired samples appear to cluster (Fig. 2B). Spatial heterogeneity has the potential to impact clinical decisions, as clinically diagnostic pTyr markers were statistically different in proximal regions at resection (Fig. 2C, 0 minute pairs), in some cases by more than 2-fold. Freezing delays can further alter phosphorylation heterogeneity, as paired timepoints often.

To identify systemic response to ischemia, affinity propagation analysis (23) was used to classify distinct clusters of pTyr sites with comparable temporal trends. Interestingly, several clusters behaved in a similar manner across patients, suggesting a set of coregulated signaling responses to ischemia (Supplementary Fig. S4 and Supplementary Table S3). One cluster characterized by rapid increase in phosphorylation within 5 minutes of cold-ischemia included MAPK12, MAPK13, MAPK14, SHC1, GAB1, and SHIP (Fig. 1E, left). These acute responders to ischemia agree with the known roles of these proteins in stress responses and MAPK signaling (27–30). A separate cluster of pTyr sites whose phosphorylation decreased with increasing ischemia time included ephrin receptors, tensins, and plakophilins. These phospho-proteins are suggestive of modulation of cell adhesion, cytoskeletal regulation, and migration-related processes, all consistent with perfusion stress (Fig. 1E, right; refs. 31–33). Other clusters include pTyr sites with minimal quantitative fluctuations, as well as sites with bidirectional regulation during the course of 60-minute cold-ischemia (Fig. 1E, middle). A 'core' ischemia-regulated phosphorylation signature containing 21 phosphorylation sites was detected across all ovarian tumor specimens. We devised a quantitative "ischemia-index" to compare the relative severity of ischemia response among this consensus set. This signature represents four broad functional categories: adhesion and migration, inflammatory response, proliferation, and stress response (Fig. 1F). Given the primary importance of these pathways in tumor biology, delayed-freezing artifacts will likely create erroneous biologic interpretations if tumor specimens intended for pTyr analysis are not immediately frozen.
Postexcision ischemia induces rapid, widespread, patient-dependent alterations to pTyr networks. A, heatmaps of all quantified peptides from individual patient-derived ovarian tumor 4-timepoint ischemia study. Rows indicate mass spectrometry-derived quantitative levels of pTyr sites in log2 scale relative to t = 0 minute, rank ordered by value at t = 60 minutes. Onset of color in the heatmaps corresponds to changes ≥ 3 SD from value at t = 0 (red, increasing; blue, decreasing). See Supplementary Table S1 for details. B, hierarchical-clustered heatmap of quantitative pTyr profiles of individual ovarian tumors at resection. Quantitative levels of pTyr sites grouped in rows, in log2 mean normalized scale. C and D, select examples of distinct inter-patient ischemia regulated temporal dynamics. Colored lines represent relative inter-patient phosphorylation levels; mean ± SD of technical replicates is shown. Temporal data values shown are derived from independent patient datasets and normalized with t = 0 value measured in B (see Supplementary Fig. S3 for additional examples). E, affinity propagation-derived clusters for pTyr sites detected in patient 27 (See Supplementary Fig. S4 for affinity propagation clusters of other patients). Subplots signify groups of pTyr sites with similar quantitative trends across time (x-axis). Solid lines denote trends of individual pTyr sites. Mean log2 temporal values relative to t = 0 minute are shown. Exemplar pTyr site of each cluster shown in blue. F, ischemia-regulated phosphorylation sites common to all patients with functional categorization. Ischemia index (y-axis) for each site represents absolute sum of the log2 changes relative to t = 0 at 5, 30, 60 minutes. Mean value ± SEM from all patients is plotted. Asterisks denote sites known to be implicated in protein activity or function.
provided different signaling profiles (Fig. 2C; 10, 30, 60 minute pairs).

Spatial and temporal protein expression differences are minimal in tumor samples

To evaluate whether intratumor protein expression heterogeneity contributed to the observed spatial and temporal pTyr differences, we performed protein expression analysis of the colorectal specimens (Supplementary Fig. S8 and Supplementary Table S5). Unsupervised hierarchical clustering indicated that protein expression is mostly uniform temporally and spatially (Fig. 3A). Where quantitative protein expression differences were observed between paired timepoints, they were muted compared with the large differences identified in pTyr profiles. To systematically evaluate the relationship between protein expression heterogeneity and pTyr levels, we identified 76 pTyr sites where corresponding protein levels were also available (Fig. 3B and Supplementary Table S6). Although protein expression was invariant with both spatial location and ischemia, pTyr levels showed dramatic spatial and ischemic time-dependent variation. These results suggest that differential ischemic responses could occur in adjacent parts of a tumor. For example, selected pTyr sites in patient 041 exhibited spatially distinct changes during ischemia, despite uniform expression of the corresponding proteins (Fig. 3C).

Ischemia alterations occur across tumor types, impacting a core set of functional classes, yet have the potential to affect biologically diverse pTyr pathways

To reduce the impact of spatial heterogeneity on phosphorylation and identify consistent ischemia-related changes, we averaged the pTyr measurements from paired-timepoint samples. As with the ovarian tumors, a large proportion of pTyr sites showed significant quantitative changes following delayed freezing (Fig. 4A and Supplementary Table S4). These results demonstrate that delayed-freezing effects can alter tyrosine phosphorylation.
phosphorylation in multiple tumor types. As with the ovarian cancer samples, inter-patient differences were observed for temporal dynamics (Supplementary Table S4). From these data, we extracted a minimal ischemia-regulated signature of 12 pTyr sites common to both ovarian and colorectal samples; adhesion and migration, proliferation, and stress response pathways are represented within this signature (Fig. 4B).

Because of the inter-patient biologic variation, many additional specimen-specific ischemia-driven pTyr perturbations were documented. Accordingly, when all tumor variation is considered, it becomes apparent that extensive ischemia-dependent alterations affect most tyrosine kinases (Fig. 4C and Supplementary Table S7). The pathways impacted by ischemia encompass diverse kinases and downstream effectors across broad biologic functions and processes (Supplementary Fig. S9). Therefore, ischemia, particularly of unknown duration, may distort pTyr network profiles to an extent that cannot be reliably corrected.

**Discussion**

There is a strong impetus to comprehensively integrate genetic profiles of tumors with corresponding proteomic expression and protein phosphorylation datasets (9). The salient findings of this study indicate that measurement of pTyr signaling nodes in human tumors is (i) susceptible to extensive postresection ischemia effects creating rapid and systemic changes that alter the initial in situ tumor phosphorylation profile, and (ii) distinct intratumor phosphorylation profiles are apparent, indicating spatial microheterogeneity and presumably signaling differences within specimens.

Our findings suggest that the tumor specimens are actively regulating signaling events despite their loss of blood supply and attachment to the surrounding tissue. Hypoxia, hypoglycemia, acidosis, hypothermia, and osmotic disturbances are perturbations to which the tumor acutely responds until cryopreservation. The immediate implication is that insight about tumor pTyr signaling in human tumor specimens may be greatly misleading or incorrect depending on the nature and duration of tumor harvesting/processing before analysis. Unfortunately, retrospective extrapolation of an accurate in vivo phosphorylation state appears to be prohibitively difficult due to unique dynamics on each pTyr site coupled with unpredictable patient specific responses to ischemia (Fig. 1C and D and Supplementary Fig. S3).

The temporal trends and directionality of pTyr fluctuation observed are consistent with the physiologic effects of tumor...
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Isoflurane and a step-wise signaling response. In fact, the pTyr sites within the identified temporal clusters appear to correlate with progressive stages of ischemic stress (Fig. 1E and Supplementary Fig. S4 and Supplementary Table S3). For example, physical stresses from wounding, hypoxia, and osmotic shock result in the immediate activation of response pathways to promote tissue repair and regeneration. This activity is consistent with the observed rapid hyperphosphorylation of p38 MAPKs (i.e., MAPK12, MAPK13, MAPK14) through oxidative stress-sensing ASK1 and osmosensing OSM (34, 35). Activation of these pathways can in turn trigger signaling programs necessary for the production of proinflammatory cytokines and tissue repair (36). This immediate signaling cluster also included increased phosphorylation of mitogenic nodes on SHC1, GAB1, and MAPK1 and MAPK3 sites to potentially initiate proliferative regeneration. Sustained ischemia times were accompanied by more gradual cellular responses. Increased phosphorylation of PRKCD, a known substrate of a caspase-3 during apoptosis, was observed across all ovarian tumors. Lack of perfusion leads to cellular dehydration, shrinkage, and distortion. Accordingly,
phosphorylation decreases on EPHA2, EPHA4, EPHB2, PARD3, PRK4, and TNS3 agree with physiologic changes in loss of cell–cell and ECM contacts (31–33). Collectively, the directionality and timing of several phosphorylation sites are consistent with the physiologic stages of severe ischemia. Of note, the core pTyr ischemia signature described overlaps with functional pathways known to be relevant in the signaling of cancer cells such as adhesion, migration, and proliferation (37, 38). Importantly, in many cases the pTyr sites annotated are directly implicated in regulating protein kinase activity and function. As such, these are not uncharacterized phosphorylation events but rather functionally relevant pTyr sites. As such, these are not uncharacterized phosphorylation events but rather functionally relevant pTyr sites with probable signaling consequences. Moreover, although phosphorylation changes of greater than 2-fold were detected during the ischemia time-course (20%–28% and 25%–48% of peptides in ovarian and colon tumors respectively; Supplementary Tables S2 and S4) and often used to prioritize biologically significant signaling changes, the magnitude of change does not always correlate with signaling significance. In fact, modest changes to phosphorylation levels may correspond to meaningful biologic results as demonstrated in the context of MEK, K-RasG12D, or EGFRVIII, where slight changes to the signaling activity have profound effects on viability and oncogenicity (39–41). As such, the subset of pTyr sites with seemingly insignificant variations may still in fact push signaling networks away from a steady state (42).

It is important to emphasize that the extensive list of ischemia-regulated pTyr sites in ovarian and colon tumors (Fig. 4C and Supplementary Fig. S9) spans a multitude of pathways and processes and are not limited to obvious stress response pathway proteins (i.e., p38 MAPKs). Although it is tempting to speculate that tumors with strong driver signaling (i.e., HER2 overexpression) could still generate pTyr signatures that overshadow ischemia-induced signatures, this is conceptually unlikely for at least two reasons. First, oncogenic signaling mutations do not always exhibit enhanced levels of phosphorylation, but can instead present persistent, minor increases to achieve oncogenic network states (40, 41). Second, strong pTyr signaling would likely impinge on the nodes and pathways affected by ischemia (i.e., proliferation, migration, adhesion, etc.) preventing definite attribution of the source. Thus, it will not be feasible for future pTyr experiments to simply exclude a subset of “ischemia susceptible” proteins and derive a quantitatively reliable dataset because the boundaries of susceptible and stable pTyr sites are not entirely clear, appear to be patient specific, and cannot be predicted a priori based on our current knowledge.

The use of surgically excised tumors in this study was paramount to recapitulate a typical biospecimen collection scenario; however, an unexpected caveat of this analysis was the realization that pTyr tumor heterogeneity exists even on a relatively proximal scale. Spatial heterogeneity of tyrosine phosphorylation may have significant impact on clinical decisions. As alluded to in Fig. 2C, monitoring of therapeutic efficacy in preclinical or clinical trials is often examined by measuring phosphorylation levels on kinases and other signaling targets. However, using pTyr as a diagnostic proxy in human specimens could pose challenging, as inadequate assessment of clinically relevant pTyr sites is possible depending on the extent of tumor spatial heterogeneity and breadth of sampling. Ideally, this variation should be acknowledged and accounted for in current and future studies to allow appropriate interpretation of pTyr levels in human tumor specimens (e.g., multiple, spatially distinct pre- and posttherapy biopsies when evaluating drug efficacy.)

A concurrent CPTAC study of the ischemia-driven effects on serine/threonine phosphorylation in tumor samples revealed complementary insights (21). Although examination of the same ovarian tumor specimens used here suggested that serine/threonine phosphorylation might be less susceptible to ischemia, perturbations were observed in approximately 6% of the approximately 9,000 pSer/pThr sites measured (based on those sites overlapping in at least 3 samples), with the majority of these sites concentrated in stress signaling pathways. This result is in contrast with this study, where perturbations were observed in 62% of 217 sites measured in ovarian tumors (based on those sites overlapping in at least 3 samples) and approximately 44% of 57 sites measured across both ovarian and colon tumors (overlap in at least three samples). These differences are likely attributable to the highly regulated nature of pTyr sites compared with pSer/pThr sites, and may be indicative of the biologic relevance of the regulated phosphorylation sites measured in both studies.

In summary, immediate freezing of human tumor specimens is necessary to minimize postsection artifacts that could confound identification of physiologic pTyr signaling networks. Intratumoral phosphorylation heterogeneity suggests that performing single biopsies of primary tumors or metastases, as is usual clinical practice, may provide erroneous or incomplete profiles of signaling systems in tumors. These data suggest that multiple biopsies, immediately flash frozen, may be necessary to accurately assess the signaling characteristics of human tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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