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Different Changes in Protein and Phosphoprotein Levels Result from Serum Starvation of High-Grade Glioma and Adenocarcinoma Cell Lines

Victor A. Levin^{*,†}, Sonali C. Panchabhai[†], Li Shen[‡], Steven M. Kornblau[§], Yihua Qiu[§], and Keith A. Baggerly[‡]

Departments of Neuro-Oncology, Bioinformatics and Computational Biology, and Blood and Marrow Transplantation, Section of Molecular Hematology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77230

Abstract

Tumor cells undergoing serum starvation in vitro partially mimic metabolically stressed cells trying to adjust to a changed environment in vivo by inducing signal transduction and gene expression so that the tumor continues to grow. Our hypothesis is that the changes in protein and phosphoprotein levels after serum starvation may reflect the adapted phenotype of the tumor, which could be targeted for therapy. We used reverse-phase protein microarrays to interrogate five high-grade glioma cell lines and seven adenocarcinoma cell lines for differences in the level of 81 proteins and 25 phosphoproteins. All cell lines were studied in the well-fed condition of growth with 10% FBS and the starved condition of 0.5% FBS. Protein expression levels were normalized to β -actin and trichotomized as increased (+1, upper 75th quartile), decreased (−1, lowest 25th quartile), or unchanged (0, others) to focus on the patterns of the biggest (and hopefully most robust) changes in protein and phosphoprotein levels. We examined these trichotomized values to better understand Starved-Fed differences among the cell lines and thereby gain better/clearer insight into the effects of serum starvation on potential cellular responses. In general, the expression of proteins and phosphoproteins 24 h after FBS starvation increased more often in glioma lines than in adenocarcinoma lines, which appeared to have fewer increased protein scores and more decreased scores. Many of the proteins increased in gliomas were downstream targets of the PTEN-PI-3 kinase-AKT, EGFR-MAPK-Stat, and transcription activator-polyamine signaling pathways. In adenocarcinomas, the expression of proteins and phosphoproteins generally increased in apoptosis pathways, while there were minor fluctuations in the other pathways above. Contrawise, gliomas become resistant to apoptosis after 24 h of serum starvation and upregulate transcription activators and polyamines more so than adenocarcinomas.

Keywords

glioblastoma; reverse phase protein array; cancer; nutritional stress

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*Author to whom correspondence should be addressed; Victor A. Levin, Department of Neuro-Oncology, Unit 431, The University of Texas M. D. Anderson Cancer Center, P.O. Box 301402, Houston, TX 77230-1402 USA. Tel: +1 713-792-8297. Fax: +1 713-794-4999. vlevin@mdanderson.org.

†Department of Neuro-Oncology.

‡Department of Bioinformatics and Computational Biology.

§Department of Blood and Marrow Transplantation.

Supporting Information Available: To reduce the size of this article and to provide complete presentation of the data, we have included additional Figures 9, 10, 11, and 12 and relevant information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Tumor growth and the ability of cancer cells to invade and metastasize to distant sites in the body are reflections of many genetic and molecular events that occur within nascent cancer cells and their microenvironment. Cancer biologists have long theorized about what defines cancer and what features are harbingers of the metastatic phenotype. Some of these features are the ability to grow in medium, the ability to grow as a three-dimensional mass, and an ability to withstand various forms of physical stress such as low oxygen tension, low substrate levels, deviation from ambient pH, and high- or low-temperature extremes. In the main, cancer cells adapt better than normal diploid cells to hostile environments whether in vitro or in vivo. In response to environmental stress, cancer cells produce cytokines to create new blood vessels (angiogenesis), alter their energy metabolism to become more efficient (anaerobic glycolysis), marshal remaining energy for locomotion to find a more hospitable location so that they might survive (invasion/metastasis), regulate their cell division, and as a group, even regulate their own existence (apoptosis/autophagy). These observed cell behaviors also serve as potential opportunities for the treatment of cancer in that they suggest the possibility of directing therapies to weaknesses in these adaptive mechanisms. Over the years, experimental therapies have been devised to counter cell division, cell invasion, metastasis, and angiogenesis and to induce apoptosis.

Gaining an understanding of cancer cells and anticipating how they will respond to stress have always been difficult. Most efforts have been made to study mechanisms of drug action or resistance from a macro level, that is, scientists have typically studied the effects of stress on gene upregulation and down-regulation, genes that are silenced by methylation, proteins that are overexpressed or underexpressed, and the like. Throughout this process, time and technology have been our enemies: patients continue to die of cancer, and in many cases, we are no closer today to cure than we were 30 years ago. This is unfortunately especially true for glioblastoma and other high-grade gliomas. High-grade gliomas are uncommon malignancies, representing less than 1.5% of all cancers and they grow, invade, and respond to nutritional deficiency in unique ways. Thus, the challenge is to understand unique target opportunities for cancer therapy that may impact the growth and spread of specific cancers. In response to that challenge, we have taken some small steps that we hope can accelerate ex vivo study of intact living cells. The first step was to develop a new inexpensive high-throughput three-dimensional assay methodology to evaluate drugs alone and in combination.^{1,2} The second step, using reverse-phase protein microarrays (RPPAs), was briefly presented in a prior study³ but is described here more fully.

We and others have studied tumor cell proteins using RPPAs, a semiautomated methodology that allows the reasonably precise study of large numbers of antibodies to proteins in extensive experiments. This methodology has the potential to rapidly determine how tumor cells respond to various types of stress and thus help guide the development of therapeutic strategies that take advantages of weaknesses in those responses. However, this type of analysis has not yet been adequately applied and tested for its ability to provide useful information to help develop treatment strategies. The current study compares five high-grade glioma and seven adenocarcinoma cell lines to determine the impact of serum starvation (simulated by culture in 0.5% fetal bovine serum [FBS]) on protein and phosphoprotein signals between the two cell types and among the individual cancer cell lines. We used RPPAs to quantitate protein and phosphoprotein changes in response to serum starvation stress and found that this methodology was reliable for rapid large-scale proteomic analysis of robust changes in protein expression and phosphorylation states. Our hope was that we would be able to define patterns by which some, but not all, cell lines adapt in order to survive and, in that process, provide information that may be helpful in developing treatment strategies based on how these cancers adapt to stress. For instance, if

protein level or phosphorylation increases in a starvation activated pathway, it might be good to inhibit proteins or phosphorylation in that pathway to magnify the impact of starvation stress on the cell and, in the process, force the cell closer to apoptosis or autophagy.

Materials and Methods

Cell Culture Lines

A total of 12 cell lines were used in this study. The seven adenocarcinomas were three human breast cell lines (MCF7, MDA231, MDA468), a gift from Francisco Esteva (M. D. Anderson Cancer Center, Houston, TX); a human pancreatic carcinoma (MiaPaCa), a gift from Kapil Mehta (M. D. Anderson); a human colon carcinoma (KM125c), a gift from Isaiah Fidler (M. D. Anderson); and two human ovarian carcinomas (OVCAR5, SKOV3), purchased from the ATCC (Manassas, VA). The five high-grade glioma lines were U87, U251HF and SNB19, bought from the ATCC, and LNZ308 and LN229, a gift from Oliver Bogler (M. D. Anderson). Cell lines were maintained in Dulbecco's modified essential/F12 medium (DMEM/F-12) supplemented with 10% FBS and 1% penicillin-streptomycin antibiotic. The cells were kept at subconfluent levels until harvest.

Antibodies and Validation

All the antibodies used in this study are listed in Table 1. One of the key challenges in working with RPPAs is the availability of high quality antibodies. As a result, to ensure that our antibodies were of sufficient quality for our RPPAs, we used a denatured protein array and confirmed the specificity of the antibodies using Western blotting.⁴ Each candidate antibody was subjected to a stringent validation procedure in which they displayed a predominant single band in Western blots against cell line lysates previously selected for testing of antibody specificity. Antibodies against phosphorylated epitopes had to show specificity against samples stimulated (e.g., with growth factors) or inhibited (with specific inhibitors) to yield phosphorylated or nonphosphorylated forms of the protein. Finally, for antibodies passing the above criteria, results using RPPAs had to parallel those seen from Western blots.

Preparation of Cell Lysates

Our goal was to study 12 cell lines under conditions of well-fed growth (that is, in cultures containing 10% FBS) and "starvation growth" (in cultures containing 0.5% FBS). The techniques used are similar to those published previously.^{3,4} Briefly, cells were plated in flasks without exceeding 10^6 cells/mL to avoid having cells enter apoptosis naturally. Cells were harvested using 0.25% trypsin and counted with a Vi-Cell Counter (Beckman Coulter), and then 5×10^6 cells were transferred to 6-well plate (35-mm diameter; 5-mL volume) and grown for 24 h at 37 °C in 5% CO₂. Cells were cultured in parallel for 24 h, under both FBS concentrations. The cells were washed twice in ice-cold phosphate-buffered saline (PBS), lysed in 30 μ L of RPPA lysis buffer (1% Triton X-100, 50 nmol/L Hepes [pH 7.4], 150 nmol/L NaCl, 1.5 nmol/L MgCl₂, 1 mmol/L EGTA, 100 nmol/L NaF, 10 nmol/L NaPPi, 10% glycerol, 1 nmol/L phenylmethylsulfonylfluoride, 1 nmol/L Na₃VO₄, and 10 μ g/mL aprotinin; alternatively, whole proteinase inhibitor tablets could be used [Boehringer/Roche, Mannheim, Germany]) for 20–30 min with vortexing on ice every 5 min, and then centrifuged for 15 min at 14 000 rpm; the supernatant then was collected. Protein concentration was determined using BCA Protein assay (bicin-choninic acid) according to manufacturer's protocol (Thermo Scientific, Rockford, IL). Lysates were transferred at volumes of 25–30 μ L (after adjusting protein concentration to 1 μ g/ μ L for each sample) into a polymerase chain reaction (PCR) 96-well plate. Ten microliters of 4 $\times$ SDS/2-ME sample buffer (35% glycerol, 8% sodium dodecylsulfate [SDS], and 0.25 mol/L Tris-HCl

[pH 6.8], with 10% β -mercaptoethanol added before use) was added to each sample well. The plates were covered and incubated for 5 min at 95 °C and then centrifuged for 1 min at 2000 rpm.

Array Assembly and Printing

The array assembly and printing procedure has been described in full previously.⁴ In brief, five serial 1:3 dilution steps were made for each protein lysate, spotted in triplicate, and arrayed in 384-well plates (Genetix, Boston, MA). There is very little variation arising in protein loading so long as we take equal volumes for each dilution since the original lysate protein concentration has been adjusted to 1 $\mu\text{g}/\mu\text{L}$. Samples were printed in triplicate onto nitrocellulose-coated glass slides (FAST Slides, Schleicher & Schuell BioScience, Inc., Keene, NH) using an Aushon Bio-sytems (Burlington, MA) 2470 Arrayer with 175- μm pins and a single touch action. For each triple, one series was located in the middle of the array and the other two were split on either side and arranged in reverse orientation, thus, allowing us to estimate and correct for spatial trends. To correct for staining, background, and loading variation across (array) slides, a positive control, a mixture of the 12 cell lines (hereafter called the “pooled control”), and a lysate buffer negative control were printed at the end of each patient sample row, creating a grid across the whole slide.

Antibody Detection and Array Staining

A detailed description of the array methodology, including antibody staining and detection, has been published.⁴ Briefly, after printing, slides were incubated for 15 min in biotin blocking solution to block endogenous peroxidase, avidin, and biotin prior to incubating slides in protein block at 4 °C overnight. Primary antibodies in concentrations from 1:100 to 1:2000 were added for 1–2 h with frequent rotation (see Table 1 for dilutions and antibody manufacturers). A biotinylated secondary antibody (anti-mouse or anti-rabbit), diluted 1:10 000–1:20 000, used as starting point for signal amplification, was added and kept in contact with the slides for 1 h. Thereafter, array slides were incubated during exposure to the DAKO (Copenhagen, Denmark) signal amplification system, which uses catalyzed reporter deposition of substrate to amplify the signal detected by the primary antibody. Slides were incubated in streptavidin–biotin–peroxidase and biotinyl tyramide/hydrogen peroxide reagents each for 15 min with frequent washing in between. Finally, 3,3'-diaminobenzidine tetrachloride was cleaved by tyramide-bound horseradish peroxidase, giving a stable brown precipitate with excellent signal-to-noise ratio. This technique is sensitive to the femtomolar range.⁴

One hundred six proteins (exclusive of the two β -actin proteins used to normalize the data) were assayed; this includes 25 phosphoproteins (Table 1), which were sought using antibodies validated as described previously.⁴ We utilized a naming system in which the protein name always came first, followed by parentheses enclosing the abbreviation for the phosphorylated amino acid.

Data Analysis and Statistics

Hybridized slides were scanned on a desktop scanner (HP, Sunnyvale CA) at an optical resolution of 1200 dpi in 16-bit grayscale and saved as TIFF files. The protein expression intensity of each spot was measured with the automated software program MicroVigene (VigeneTech, Inc., North Billerica, MA). Because we tested a dilution series of samples, we were able to develop a dilution-concentration-expression curve providing relative expression intensities. All analyses were performed using the R Statistical Programming Environment, version 2.4.0.

Quantification and Normalization

Array images were produced using ImageQuant software (GE Healthcare, Chalfont St. Giles, U.K.), and individual spot values were summarized using the MicroVigene (VigeneTech) RPPA module. Spots from an array were adjusted for spatial variation in background staining using topographic normalization. This approach takes advantage of the positive and negative controls across the whole slide to yield more reproducible replicate spots and to match known dilution steps more closely. Neeley and colleagues (Neeley, S., Baggerly, K. A., and Kornblau, S. M., submitted 2009) provide a more detailed description of the algorithm. In brief, a generalized additive model is fitted to the log intensities of all positive controls over the surface of the array. Then, each series is mapped to the spatially closest positive control and all log values in that series are replaced with [raw value + (median control – local control)]. This procedure “flattens out” the spatial variation. After preprocessing, the R package SuperCurve 0.931 (available at <http://bioinformatics.mdanderson.org/Software/OOMPA>; accessed on February 9, 2009) was used to summarize each five-step dilution series into one single log-scale protein concentration value representing the midpoint of the dilution curve. The algorithm implemented fits a joint four-parameter logistic model.^{5,6} In all, 111 arrays were run (one antibody per array). The arrays were produced in two batches, with 47 slides printed in the first batch and the rest in the second. Since batch effects were expected, we corrected for them by running a β -actin slide in both batches and then analyzing all sample concentrations relative to the β -actin concentration for that sample in that batch (the two β -actin slides are not counted in the 111 slides). β -Actin was chosen because of its stability and consistency in the treated and untreated environments and the fact that its protein, mRNA, and gene expression are widely used to normalize biological data. β -Actin, however, is not without shortcomings as it can increase with cell division and other biological events.^{7–9} Nonetheless, since we assume little increase in cell proliferation for a given tumor cell line during the 24 h of study, we believe that until a better normalization protein/process is available the β -actin normalization will have to suffice. After correcting for batch effects by subtracting the β -actin summary values from the summarized values of all proteins, the median batch-corrected value of the three replicates was chosen to represent the protein expression in each cell line. Of the 111 arrays, five were “duplicates,” in which the same antibody was used. In the final analysis, the pairs of duplicate arrays were inspected visually and the “cleaner” images (that is, those for Akt, BID, NPM, p16, and PKC δ) were used, providing a set of 106.

Results

Analysis of RPPA Data

While our tools yield quantitative estimates, evaluation of subtler effects depends on the precise analysis and processing methods employed. In this study, we were more interested in focusing on larger, methodology-robust changes, and our analysis methods are consequently fairly coarse. For the benefit of others who may wish to explore the finer details more fully, we have posted the raw data (spot and dilution series quantifications) at <http://bioinformatics.mdanderson.org>. We first sorted expression levels of 106 proteins across all cell lines available as log-scale midpoint dilution curve values. We then assessed the distribution of these values throughout the matrix to focus our attention just on the largest differences (and to avoid being sidetracked by noise) by checking histograms and quantile-quantile (Q-Q) plots. The quantile plots (not shown) revealed that the data were roughly normally distributed. We then assigned the highest 25% of the values a score of +1, the lowest 25% a score of –1, and those between 25% and 75% a score of 0. The quartile cutoffs were chosen arbitrarily. This trichotomization helped us focus on the largest and most visibly reproducible changes between cell lines. Each cell line was then assigned a

“score” that was the sum of the scores (−1, 0, +1) for its component antibody values standardized by dividing by its standard deviation (described in next paragraph). In assessing the variability of the above scores, we need to account for the fact that the levels of different antibodies are not independent; what can vary is the specific subset of proteins we choose to examine. We capture this type of variability using bootstrapping as follows. One hundred six antibodies were randomly chosen, with replacement, from the set of those available; values from this new set were trichotomized as above, and the summary scores were computed. Such resamplings were repeated 999 times. These values were used to obtain data-specific bootstrap standard deviations for the score of each cell line, and the scores were divided by these standard deviations to produce standardized values. Hierarchical clustering was also applied to the 12 cell lines based on the “robustified” score matrices defined above using Euclidean distance with complete linkage.

Bootstrapping assembles “pseudo” data sets by choosing entire slides at random with replacement. To an extent, this parallels the process of choosing a specific set of proteins at which to look. Since all of the measurements for a given protein are retained or not as the slide is sampled, the extra variation in the scores introduced by the dependence between measurements is modeled correctly. Though our specific tests involve bootstrapping, we would like to provide some rough intuition as to what types of sums should be considered “large”. To do this, we note that the standard deviation of the sum assuming independence is 7.31 units, so differences of 20 or more units are likely to be significant, even after accounting for dependence of the scales we are encountering here. This approach of specifically trichotomizing into “up a lot”, “down a lot”, or “not changed a lot”, has not been published previously; we invented it for this analysis where we designed a statistic to capture the characteristic of interest (gross change) and then used simulations (via bootstrapping) to calibrate the behavior of this statistic.

Well-Fed Cell Lines

We created a data matrix of 106 antibodies for all the cell lines in their original well-fed state in 10% FBS. Expression levels of 106 proteins across all cell lines were normalized with β -actin, sorted, robustified, and standardized as mentioned earlier. Figure 1 shows the box plots for the bootstrapped total standardized antibody scores for each cell line, with outliers shown as circles. Using this plotting method, most cell lines looked similar, with the possible exceptions of SNB19, U251, MiaPaCa, and LNZ308. The median expression values for all the proteins in the well-fed cell lines were close to zero, with no considerable changes occurring in protein expression. Figure 2 shows the results of a hierarchical cluster analysis, which we used to determine if cell lines could be grouped with respect to normalized antibody scores. As with Figure 1, the gliomas failed to cluster as a group, meaning that they are more or less similar and protein levels were uninformative in well-fed cells.

Effect of Serum Starvation

To test the effects of serum starvation on protein and phosphoprotein level, we created a data matrix of the 106 antibodies for all the cell lines after 24 h of starvation (starved state) and then subtracted the expression values obtained for the well-fed cells. With the use of protein levels, the postactin differences were sorted, “robustified,” and standardized as described earlier. Table 2 shows the scoring for all cell lines and all normalized antibodies. The sum of scores for each protein and phosphoprotein was interpreted as follows: the lowest sum represents the greatest reduction and the highest sum the greatest increase in the 106 protein signals. The cell lines with the highest sums were glioblastoma lines LNZ308 (sum = 60) and SNB19 (sum = 40), while two with the lowest sums were adenocarcinoma lines MCF7 (breast; sum = −45) and SKOV3 (ovary; sum = −35). This more than 80-point

spread demonstrates the extremes among cell lines with respect to protein and phosphoprotein levels support major differences in the ways cancer cells respond to the stress of protein starvation. This was further supported by using this same matrix to create a box plot of 106 antibodies in the “starved-fed state” (Figure 3) after standardizing as described above and to produce a hierarchical cluster analysis (Figure 4). In both plots, there appears to be general separation between high-grade glioma and adenocarcinoma cell lines, along with appreciable changes in the level of proteins in the starved-fed state.

To evaluate the impact of phosphorylation and caspase cleavage, we combined the starved-fed cell-line differences from both slide batches. So we could consider the phosphoprotein and cleaved protein groups of antibodies without counting them twice, we adjusted the modified forms by subtracting the unmodified values from the initial values and then centered the data on the median value. Applying the same statistics, we created box plots of the standardized scores for each cell line (Figure 5). These plots show an almost 10-standard deviation difference between LN2308 and SNB19 cells compared to SKOV3 and MCF7 cells. Euclidean hierarchical cluster analysis of this data (Figure 6), however, did show proximity pairing of SKOV3 and MCF7 cells, while the distance separation to the paired LN2308 and SNB19 cells was not as dramatic as might be expected from Figure 5.

Taking these analyses further, we utilized the robustified starved-fed differences in protein/phosphoprotein levels from Table 2 and calculated a starved-fed “score ratio” (SR) for each cell line, where $SR = (\text{number of } +1 \text{ scores})/(\text{number of } -1 \text{ scores})$. With this equation, SR varied in the gliomas from a high of 21 in LN2308 cells to 5.4 in SNB19 cells, 2.5 in U87 cells, 2 in U251 cells, and 1.1 in LN229 cells after serum starvation. The adenocarcinoma lines behaved quite differently, with fewer high protein scores and more low scores; SR ranged from 0.9 and 0.8 in MDA231 and KM125c cell lines to 0.7 in OVCAR5 cells, 0.4 in MiaPaCa, 0.2 in MCF7 and MDA468 cells, and 0.3 in SKOV3 cells.

To test whether we were unfairly giving certain proteins too much weight in the analysis by including values for both their modified and unmodified forms, we reran the analysis with values for all modified forms omitted. The results were essentially identical, so any overweighting present does not affect our conclusions (data not shown).

Discussion

This study nicely shows many of the varied differences in protein level and protein phosphorylation that high-grade glioma cells produce in monolayer culture compared to adenocarcinoma cells when these cell lines are exposed the stress of serum starvation. The high-grade gliomas, as a group, increase protein signals and appear to become resistant to apoptosis after 24 h of serum starvation; adenocarcinomas, as a group, tend to decrease protein signals and appear more vulnerable to apoptosis. For high-grade gliomas, there is also an increase in target receptor to intracellular downstream signaling and transcription activator and polyamines pathways.

Cancer cells have been and will continue to be studied in monolayer culture, three-dimensional culture, orthotopic animal models, genetically engineered animal models, and analysis of human tumor tissue. Gains have been made in the understanding of the genetic basis for tumor causation through the study of DNA and RNA. All these analytical techniques have engendered methodologies to interrogate the signal and transcriptional control of cancer growth and methodologies to help discriminate tumors' histological features and, to an extent, ability to respond to certain chemotherapy agents.^{10,11} Proteins, and in particular modified proteins such as phosphoproteins, cleaved proteins, and protein isoforms, are more difficult to study in the breadth required. That is, while DNA arrays and

FISH can study thousands of genes for presence, translocation, amplification, and the like, the complex nonlinear interactions of cellular proteins that lead to and support the various tumor cell phenotypes (signaling activation and deactivation, phosphorylation state, expression and timing of expression relative to other cell functions, modification that produces breakdown or unique isoforms, and so on) are still difficult to study at the scale needed for complete understanding.

As a first approximation of protein and phosphoprotein levels, we determined the antibody level and normalized it to antibody to β -actin as a general measure of cell size and, therefore, protein content. We then calculated the starved-fed values for each of the 106 validated proteins in 12 cell lines. We then determined which cells had negative starved-fed values (below the 25th quartile, designated -1) and which had positive starved-fed values (above the upper 75th quartile, designated +1). We now explore what understanding can be gleaned from these data, which are summarized in Table 2 and Figure 7. First, it appears that under serum starvation conditions glioma lines increase their protein expression and phosphorylation to a greater extent than adenocarcinoma lines. On average, three of the glioma line (LNZ308, SNB19, U87) average +29, while the lowest four adenocarcinomas (MiaPaCa, MDA468, SKOV3, MCF7) average -20 scores. One could argue that differences among the glioma lines are less than those among the adenocarcinomas representing four organ-specific carcinomas: ovarian (OVCAR3, SKOV3), breast (MCF7, MDA231, MDA468), pancreas (MiaPaCa), and colon (KM125c). As shown in Table 2, SR varied from highs of 21 and 5.4 in LNZ308 and SNB19 glioma cells to lows of 0.2 in MDA468 and MCF7 breast adenocarcinoma cells. These SR measures clearly show the propensity of high-grade glioma cells to increase their protein and phosphoprotein levels in response to serum starvation stress, while adenocarcinomas respond with a reduction in protein and phosphoprotein levels.

In addition to looking at increases and decreases in phosphoproteins, we were concerned about the extent to which results for the phosphorylated forms were driven by differences in abundance of the base form of the protein. To investigate this, we examined how our results differed when we (a) used values for the modified proteins directly and (b) used values after adjusting for baseline abundance. Since the values we use are on a log scale, this adjustment simply involves subtracting the baseline values from the modified values before pursuing trichotomization and later analysis. While our aggregate results remain robust, clear evidence that adjusting for baseling is an important step is provided by simple examination of the fraction of trichotomized values that shift (Figure 8). Very few of the baseline scores change due to adjustment pushing a few into the top or bottom quarter; only 8/228 total comparisons (3.5%) show changes. The distribution of extremes among the modified forms, however, shifts quite a bit, with 104/252 comparisons (41.3%) showing changes. As shown in Figure 8, where the trichotomized values both before and after baseline adjustment are collated, it can be seen that trends of increased phosphorylation attributed to gliomas lines and decreased phosphorylation attributed to adenocarcinoma lines may be overstated when correcting for the change in base protein levels. This appears to be specifically true in LNZ308, MDA468, SKOV3, and MCF7 cell lines where original phosphoproteins sum scores were too high (LNZ308) or too low (MDA468, SKOV3, MCF7) when compared to the corrected phosphoproteins value.

How best to utilize data such as ours will not be answered by this study and may ultimately require greater knowledge and understanding of quantile phosphoprotein signals per unit time and distance. Nonetheless, not to interrogate the implications of our protein and phosphoprotein data would, we believe, be a mistake for the readers of this paper. Therefore, in an effort to understand the implications of these differences on the biology of gliomas and adenocarcinomas as they try to survive the stress of serum starvation, we examined the

impact of these protein changes on some known and theoretical signaling pathways. Despite the limitations of pathway analyses and our ability to fully understand the many nonlinear and intersecting cellular signals involved in them, using a number of systems and pathway publications we arrived at consensus pathway proteins for four groups of pathways: the PTEN-PI-3 kinase-Akt pathway (Supplement Figure 9), apoptosis pathways (Supplement Figure 10), EGFR-MAPK-Stat pathway (Supplement Figure 11), and transcription activator-polyamine pathways (Supplement Figure 12). By reviewing the known features and activities modulated by these pathways, we were able to make some inferences that may be helpful in designing future therapeutic interventions. While cell lines within each superfamily (glioma and adenocarcinoma) varied, to generalize our observations about the pathways identified here, we emphasize those protein changes seen in more than 50% of the cell lines in each tumor superfamily in the discussion that follows.

For PTEN-PI-3K-AKT pathway proteins, one glioma line (LNZ308) showed a sum score of +11, while four adenocarcinoma lines showed an average -6 decrease in protein level sum scores (Supplement Figure 9). The glioma lines showed upregulation of Akt(Ser473), Akt(Thr308), FoxO3a(Ser318/321), NF- κ B(Ser276), PI-3 kinase, PKC β 1, and PKC α (Ser657); the only downregulated phosphoprotein in the glioma lines was p70 S6 kinase (Thr389). This is in contradistinction to the adenocarcinoma lines, in which PKC α and PTEN were increased and Akt, Gab2, PKC β 1, and survivin proteins were decreased. The consequences of this may be that PI-3-kinase and phosphorylated Akt occur where PTEN is mutated and this will then activate downstream proteins like NF- κ B, FOXO3A, and Bad, which are phosphorylated and thus evade apoptosis and promote cell survival.¹² On the other hand, in the case of adenocarcinomas, the PI-3 kinase-AKT pathway is inactive, leading to decreased levels of AKT and PI-3 kinase; this coupled with decreased survivin, a negative regulator of apoptosis that inhibits caspase activation,¹³ leads to decreased cell survival. Moreover, the level of Gab2, a principal activator of PI-3 kinase,¹⁴ is also decreased in adenocarcinomas, promoting decreased survival. The only molecule of the PI3 kinase-AKT pathway that is not increased in gliomas is p70 S6 kinase, the levels of whose phosphorylated form in fact decrease in gliomas. The explanation for this could be that low nutrient levels or growth factor reduction can inhibit mTOR and that p70 S6 kinase, a target of mTOR, is thereby inhibited, leading to decreased levels after serum starvation. If so, then glioma cells may be much more resistant to serum starvation than adenocarcinoma cells and continue to survive and proliferate, while they also keep at hand the mechanism capable of inducing autophagy and protecting the cells until the stress is overcome. In short, gliomas appear better able to resist protein starvation stress than adenocarcinomas, in part by evading apoptosis better.

Protein levels increased in the apoptosis pathway in the majority of serum starved glioma lines on average by +10 with LNZ308 and SNB19 lines averaging 18, while on average adenocarcinoma lines decreased their sum score to -8 with SKOV3 and MCF7 lines averaging -14. We believe that in glioma lines this would hinder apoptosis (Supplement Figure 10) through increased Bax, Bcl2, Bcl-xL, caspase 3, cleaved caspase 7(Asp198), CDK4, cyclin B1, HDAC 3, MDM2, p16, and p21; reductions occurred in only the GSK-3 α/β (Ser21/9) protein. Adenocarcinomas again differed from the glioma lines, with increases in p53 protein and decreases in the Bad(Ser155), Bcl-xL, CDC2, CDK2, cIAP-1, HDAC 3, YAP, β -catenin, and β -catenin(Ser33/37/Thr41) proteins. Starvation-induced protein expression in gliomas appears to serve an antiapoptotic function, as suggested by the increases in Bcl2, Bcl-xL, and cIAP. This conclusion is also supported by the fact that in gliomas there was an increase in MDM2, which is a protein responsible for degrading p53,¹⁵⁻¹⁷ while in adenocarcinomas there was an increase in the level of p53, which would be likely to trigger p53-dependent apoptosis in these cells. The activity of cyclin-dependent kinases (e.g., CDK4) and cyclins (e.g., cyclin B) also increased in the gliomas, and this

would be expected to inhibit Rb;^{18,19} indeed, we found decreased levels of Rb in gliomas. Moreover, our finding that the levels of CDC2 and CDK2 decreased in the adenocarcinomas suggests that these cells would be encouraged to undergo cell cycle arrest. Also important is the Wnt signaling pathway, wherein the phosphorylation of Akt inhibits GSK-3^{20–22} such an event was evidenced by the decreased levels of phosphorylated GSK-3 in the gliomas, while in adenocarcinomas there appears to be increased sequestration of β -catenin (that is, a decrease in the level of phosphorylated β -catenin). Thus, our evidence supports the notion that Wnt signaling may be active in gliomas and inactive in the adenocarcinomas studied.

Our quantification of protein and phosphoprotein expression included the interrogation of several members of the EGFR-MAPK-Stat pathway (Supplement Figure 11). In gliomas we found inconsistency among the cell lines with increases in sum scores of +20 in LN2308 and +12 in SNB19 but falling to a –4 sum score in LN229 glioma lines. In adenocarcinoma lines, we also saw great variability in scores with the greatest decrease being –16 in MCF7 cells. Interpreting the implications of protein sum scores is difficult. In glioma lines, it appeared that increases in AMPK α (Thr172), CD49b(integrin α 2), ERK1/2(Thr202/Tyr204), Erk2, FoxO3a(Ser318/321), HER-2/ERBB2-(Tyr1248), HER-3/ERBB3, Jab1, MEK1/2, Rac1/2/3, Stat1, and Stat3 proteins were seen, while only Stat5 decreased. In contrast, most of the adenocarcinomas showed no increases in protein or phosphoprotein levels and decreases in only HOXA10 and MEK1/2(Ser217/221).

We combined results for members of transcription activator and polyamine pathways since polyamines can enhance transcription.²³ On average, four glioma lines increased sum scores to +6 (LN229 was +1), while adenocarcinoma line sum scores were more variable with SKOV3 and MCF7 averaging –8 (Supplement Figure 12). In general, gliomas showed increases in ADOMET, antizyme inhibitor, Erg-1/2/3, Jab1, MSI2, Nur77, Rac1/2/3, and ZNF342. In most gliomas, no proteins were decreased. Protein and phosphoproteins differed markedly in adenocarcinomas, with no protein increases and decreases in only ELK-1(Ser383) and c-Myc. Thus, in gliomas, it appears that the protein level of Erg, a sequence-specific transcriptional activator,²⁴ goes up. In addition, Elk1, a nuclear target for the ras-raf-MAPK-pathway,²⁵ also increases; Jab1, an important effector of a novel signal transduction pathway for PAR-2,²⁶ and MSI2, an RNA binding protein that regulates the expression of target mRNAs at the level of translation,^{27,28} also are increased. In addition, the orphan nuclear receptor Nur77, which is thought to help cells evade apoptosis,^{29,30} appears to be increased in gliomas. We found that ornithine decarboxylase (ODC), the initial polyamine promoting proliferation and transcription,²³ was not increased in gliomas, but that the ODC antizyme inhibitor that decreases ODC degradation was increased as was SPMS, a rate limiting step in polyamine catabolism, which was increased in the gliomas. The effect of increased antizyme inhibitor and SPMS would be an increase in polyamines without expression of additional ODC protein.²³ The observed increase in ZNF 342, a transcriptional factor,³¹ in gliomas is also consistent with these cells' proliferative response to starvation stress. Most of these proteins remained unchanged in the adenocarcinomas we studied, and the level of C-myc, which is involved in proliferation,^{32–34} in fact decreased here. Taken together, these findings indicate that much more intracellular activity occurs in gliomas than in adenocarcinomas.

Caveats and Extensions

We have used our results for these 12 cell lines and 106 proteins to draw broader inferences about gliomas in general. In particular, we have identified and enumerated the proteins in our set associated with four canonical pathways, and have used trends we see in the robust measures of expression difference we use to infer how these pathways are affected. These are our inferences.

We believe that more might be learned from our study of 106 proteins in 12 cell lines through the application of more sophisticated modeling methods. We, therefore, provided the full database as Table 2 and will supply our Microsoft Excel file of the raw data values on request to other investigators. However, the current studies tell us enough to know that high-grade glioma lines differ, in general, from most adenocarcinomas with respect to their response to serum starvation stress and that within the glioma and adenocarcinoma groups there is also some heterogeneity of protein and phosphoprotein levels. To extend these studies and to better understand and anticipate weaknesses in cells' responses to stress, we plan to study how these same tumor cell lines deal with hypoxic stress when grown in monolayer and in three-dimensional culture and to investigate the effects of specific signaling inhibitors on their adaptation to stress. We believe that studies such as these, which highlight the molecular mechanisms of adaptation to various stresses,³⁵ will provide a tool for scientists to develop better strategies in treating human cancers one day.

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Figure 1.

Standardized scores representing overall levels of protein expression in 12 cell lines (blue = glioma, red = adenocarcinoma). Cells were grown in a “fed” environment including 10% FBS and harvested during the exponential growth phase before 80% confluence was reached. Scores were obtained as follows: Expression levels of proteins and phosphoproteins detected by 106 antibodies and normalized for β -actin across all cell lines were sorted and “robustified”—the highest 25% of the values received a score of 1, the lowest 25% a score of -1, and others a score of 0. The score for a cell line is the sum of the scores for its component expression values, standardized by dividing by its bootstrapped standard deviation. The F at the end of the cell line signifies fed cells.

Figure 2.

Euclidean hierarchical clustering of the 12 cell lines based on the robustified fed condition protein and phosphoprotein expression levels detected by 106 antibodies. This clustering uses the data matrix described in Figure 1. Blue = glioma; red = adenocarcinoma. The F at the end of the cell line signifies fed cells.

Figure 3. Standardized scores representing starved-fed differences in protein expression for 12 cell lines. Protein and phosphoprotein levels detected by 106 antibodies were obtained from cells in both fed and starved conditions, and the starved-fed log scale differences were recorded and then robustified and standardized as in Figure 1. Blue = glioma; red = adenocarcinoma.

Figure 4. Euclidean hierarchical clustering of the 12 cell lines based on the robustified starved-fed protein and phosphoprotein expression differences detected by 106 antibodies. This clustering uses the data matrix described in Figure 3. Blue = glioma; red = adenocarcinoma.

Figure 5.

Standardized scores representing starved-fed differences in protein expression for 12 cell lines as in Figure 3 but modified so that different values were used for the 21 antibodies targeting phosphorylated or cleaved protein forms where the nonphosphorylated or intact protein was also available, in which case the raw log expression values were replaced with those for their ratios relative to the total form. Blue = glioma; red = adenocarcinoma.

Figure 6. Euclidean hierarchical clustering of the 12 cell lines based on the robustified starved-fed protein and phosphoprotein expression differences detected by 106 antibodies. This clustering uses the data matrix described in Figure 5. Blue = glioma; red = adenocarcinoma.

Protein	LN2308	SNB19	U87	U251	LN229	>50% glioma	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MCF7	>50% adenocarcinoma
Bcl-xL	1	1	0	1	0	1	1	0	-1	-1	-1	-1	-1	-1
HDAC 3	1	1	0	1	0	1	-1	-1	0	-1	0	-1	-1	-1
PKC β	1	1	0	1	-1	1	-1	0	-1	0	-1	-1	-1	-1
Erk1/2(Thr202/Tyr204)	0	1	1	-1	1	1	-1	1	1	-1	0	1	-1	1
Akt(Thr308)	1	-1	-1	1	1	1	0	-1	-1	0	-1	0	0	0
AMPK α (Thr172)	1	1	1	0	-1	1	-1	0	1	-1	0	0	0	0
Bax	1	1	1	-1	1	1	0	1	-1	0	0	0	0	0
Cleaved Caspase-7(Asp198)	1	1	0	0	1	1	0	0	1	0	0	1	0	0
Caspase-3	1	1	0	-1	1	1	0	0	0	1	0	0	-1	0
Cyclin B1	1	1	1	0	0	1	1	0	-1	0	0	0	-1	0
Erg-1/2/3	1	0	1	0	1	1	0	0	0	-1	0	-1	0	0
Erk 2	1	1	0	0	1	1	1	0	0	1	0	-1	0	0
FoxO3a(Ser318/321)	0	1	1	1	0	1	1	-1	1	0	0	0	0	0
HER2/ERBB2(Tyr1248)	1	1	-1	1	-1	1	0	1	0	-1	0	0	-1	0
HER3/ERBB3	1	0	1	0	1	1	0	1	0	-1	0	0	-1	0
Jab1	1	1	1	0	0	1	0	-1	-1	0	0	0	0	0
MDM2	1	1	1	0	0	1	0	0	0	-1	0	-1	-1	0
MEK1/2	1	1	0	1	0	1	0	0	-1	0	0	-1	-1	0
MSI2	1	1	1	1	-1	1	0	0	-1	0	0	-1	0	0
NF- κ B p65(Ser276)	1	1	0	-1	1	1	0	0	0	0	0	0	-1	0
Nur77	1	0	1	1	0	1	0	0	-1	0	0	0	-1	0
p21	0	1	1	-1	1	1	0	0	1	-1	1	0	0	0
PI-3 kinase	1	1	1	0	0	1	0	0	-1	0	0	-1	-1	0
Rac1/2/3	1	1	1	0	0	1	0	0	0	0	0	0	-1	0
SPMS	1	1	0	1	0	1	-1	0	-1	0	0	-1	0	0
Stat1	1	1	1	0	-1	1	0	0	0	0	0	0	1	0
Stat3	1	1	1	0	-1	1	0	0	0	0	0	0	0	0
ZNF342	1	1	1	0	0	1	0	0	-1	0	0	0	0	0
ADOMET	1	1	1	0	0	1	-1	0	0	0	1	-1	-1	
Akt(Ser473)	1	-1	1	1	-1	1	1	-1	0	0	-1	0	-1	
Bcl2	1	1	1	-1	1	1	-1	1	1	-1	0	-1	1	
CD49b(Integrin α_2)	1	1	1	0	1	1	-1	0	0	-1	1	-1	1	
CDK4	0	1	1	-1	1	1	1	-1	1	0	0	-1	-1	
p16	1	1	1	1	-1	1	1	1	0	0	-1	0	-1	
PKC α (Ser657)	1	1	1	1	-1	1	0	0	1	-1	-1	-1	1	
Bad(Ser136)	0	0	1	0	0	0	-1	-1	-1	0	0	-1	-1	-1
Bad(Ser155)	1	0	0	1	0	0	0	-1	0	-1	-1	0	-1	-1
β -Catenin	0	0	0	0	0	0	0	0	-1	-1	-1	-1	-1	-1
β -Catenin(Ser33/37/Thr41)	0	0	-1	0	-1	0	-1	0	0	-1	-1	-1	-1	-1
CDC2	0	1	1	0	0	0	1	-1	0	-1	-1	-1	-1	-1
CDK2	1	0	1	0	0	0	-1	0	-1	-1	0	-1	0	-1
Elk-1(Ser383)	0	0	0	0	0	0	0	-1	0	1	-1	-1	-1	-1
HOXA10	0	0	0	0	-1	0	0	1	-1	-1	-1	-1	0	-1
MEK1/2(Ser217/221)	0	0	0	-1	1	0	-1	0	0	-1	-1	1	-1	-1
c-Myc	0	0	-1	0	0	0	-1	-1	0	-1	-1	-1	0	-1
Survivin	1	0	0	0	1	0	0	0	-1	0	-1	-1	-1	-1
YAP	0	0	1	1	0	0	0	-1	0	-1	0	-1	-1	-1
PKC α	0	0	0	-1	1	0	1	1	1	-1	-1	0	1	1
SSAT	1	1	0	0	0	0	1	1	0	0	1	1	-1	1
GSK-3 α/β (Ser21/9)	0	-1	-1	-1	1	-1	-1	0	-1	0	-1	0	1	
p70 S6 Kinase(Thr389)	0	-1	-1	0	-1	-1	0	0	0	-1	-1	1	1	
Stat5	1	0	-1	-1	-1	-1	0	0	-1	1	0	1	-1	
Akt 1	1	0	0	-1	0	0	1	-1	-1	-1	-1	-1	-1	-1
cIAP-1	0	1	0	1	-1		0	0	-1	0	-1	-1	-1	-1
Gab2	1	-1	-1	0	1		0	-1	1	0	-1	-1	-1	-1
p53	-1	-1	1	1	0		1	0	1	1	-1	1	1	1
PTEN	0	1	0	-1	1		-1	1	1	1	1	0	0	1
Sum Score	37	28	21	4	5	32	-4	-2	-9	-18	-17	-21	-22	-13

Figure 7.

This table contains 57 of 106 proteins and/or phosphoproteins from Table 2 that changed in the same direction (+1 or -1) more than 50% of the time in five glioma (3/5) or seven adenocarcinoma (4/7) cell lines. To ease viewing, a modified heat map was constructed for +1 (red), -1 (blue), and 0 (green) scores. The table was sorted by the >50% glioma scores.

Protein	LNZ308	SNB19	U87	U251	LN229	Sum score	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MC77	Sum score
AKT	1	0	0	-1	0	0	0	1	-1	-1	-1	-1	-1	-4
AKTp473	1	-1	1	1	-1	1	1	-1	0	0	-1	0	-1	-2
AKTp473.**	-1	-1	0	1	-1	-2	0	-1	0	0	0	1	1	1
AKTpT308	1	-1	-1	1	1	1	0	-1	-1	0	-1	0	0	-3
AKTpT308.**	-1	-1	-1	1	0	-2	0	-1	0	0	1	1	1	2
AMPKa	0	0	0	1	0	1	0	0	1	0	-1	-1	-1	-2
AMPKaphos	1	1	1	0	-1	2	-1	0	1	-1	0	0	0	-1
AMPKaphos.**	1	0	1	0	-1	1	0	0	-1	-1	1	0	1	0
BAD	1	0	0	0	0	1	0	0	1	0	0	-1	-1	-1
BADp136	0	0	1	0	0	1	-1	-1	-1	0	0	-1	-1	-5
BADp136.**	0	0	1	1	0	2	0	0	-1	1	0	0	-1	-1
BADp155	1	0	0	1	0	2	0	-1	0	-1	-1	0	-1	-4
BADp155.**	0	0	0	1	0	1	0	-1	0	0	-1	1	1	0
CateninB-phos	0	0	-1	0	-1	-2	-1	0	0	-1	-1	-1	-1	-5
CateninB-phos.**	1	1	-1	0	0	1	0	0	1	1	0	-1	-1	0
Catenin-B	0	0	0	0	0	0	0	0	-1	-1	-1	-1	1	-3
EGFR	1	0	0	1	-1	1	1	0	0	0	-1	-1	-1	-2
EGFRp992	1	0	1	0	-1	1	0	0	0	0	1	-1	-1	-1
EGFRp992.**	0	0	1	-1	0	0	-1	0	0	0	1	0	0	0
ERK2	1	1	0	0	1	3	1	0	0	1	0	-1	0	1
ERK2p	0	1	1	-1	1	2	-1	-1	1	-1	0	1	-1	0
ERK2p.**	-1	1	1	-1	1	1	-1	0	1	-1	0	1	-1	-1
FOXO3A	1	0	1	0	0	2	1	-1	0	-1	0	0	-1	-2
FOXO3AP	0	1	1	1	0	3	1	-1	1	0	0	0	0	1
FOXO3AP.**	-1	0	0	1	0	0	0	0	0	0	-1	0	0	-1
GAB2	1	-1	-1	0	1	0	0	-1	1	0	-1	-1	-1	-3
GAB2p	0	0	1	0	0	1	1	-1	0	-1	0	0	-1	-2
GAB2p.**	-1	1	1	0	-1	0	0	-1	-1	1	0	1	0	0
GSK3	1	1	0	0	0	2	0	0	-1	0	-1	0	-1	-3
GSK3A-Bphos	0	-1	-1	-1	1	-2	-1	0	-1	0	-1	0	1	-2
GSK3A-Bphos.**	0	-1	0	-1	1	-1	0	0	0	0	0	1	1	2
HER2	-1	1	0	0	-1	1	0	0	1	0	0	-1	-1	-1
HER2p	1	1	-1	1	-1	1	0	1	0	-1	0	0	-1	-1
HER2p.**	1	1	-1	1	-1	1	0	1	-1	-1	0	1	-1	-1
MEK	1	1	0	1	0	3	0	0	-1	0	0	-1	-1	-3
MEKp	0	0	0	-1	1	0	-1	0	0	-1	-1	1	-1	-3
MEKp.**	0	-1	1	-1	1	0	0	0	1	-1	0	1	-1	0
P38	0	0	1	0	0	1	-1	0	0	0	0	-1	-1	-3
P38p	1	0	-1	0	-1	-1	-1	0	0	0	-1	1	-1	-2
P38p.**	0	0	-1	1	-1	-1	-1	1	0	0	-1	1	0	0
PKCa	0	0	0	-1	-1	0	1	1	1	-1	-1	0	1	2
PKCaphos	1	1	1	1	-1	3	0	0	1	-1	-1	-1	1	-1
PKCaphos.**	1	1	1	1	-1	3	-1	0	0	-1	0	-1	1	-2
PKC-delta	0	0	1	0	0	1	0	0	0	-1	1	0	0	0
PKCdeltap645	1	0	-1	0	0	0	0	0	-1	-1	-1	0	0	-3
PKCdeltap645.**	1	0	-1	0	0	0	0	0	0	0	-1	1	1	1
SRC-C	0	0	0	1	-1	0	0	0	0	0	-1	-1	0	-2
SRCp527	1	0	0	1	-1	1	0	0	0	0	-1	1	-1	-1
SRCp527.**	1	0	0	0	0	1	0	1	0	0	0	1	-1	1
STAT3	1	1	1	0	-1	2	0	0	0	0	0	0	0	0
STAT3phos	1	0	1	0	0	2	1	-1	0	1	0	-1	-1	-1
STAT3phos.**	0	-1	1	0	0	0	1	-1	0	1	0	-1	0	0
YAP	0	0	1	1	0	2	0	-1	0	-1	0	-1	-1	-4
YAPp	0	0	0	1	-1	0	0	0	-1	0	-1	-1	0	-3
YAPp.**	0	1	-1	0	0	0	0	-1	1	0	-1	1	1	1
Original phosphoprotein (n=19) sum score	11	2	3	5	-5	16	-3	-5	-1	-8	-10	-2	-10	-39
Corrected phosphoprotein (n=19) sum score	1	1	2	4	-3	5	-3	0	-2	-2	0	6	3	2

Figure 8.

This figure includes 17 proteins for which we also have phosphorylated states as presented in Table 2 and Figure 7. In addition, we include the Starved-Fed differences in five glioma cell lines and seven adenocarcinoma cell lines for these phosphorylated proteins showing the trichotomized values after baseline adjustment (**). To ease viewing, a modified heat map was constructed for +1 (red), -1 (blue), and 0 (green) scores. Provide is the sum score for the 19 phosphoproteins without baseline correction for unphosphorylated protein and the same 19 phosphoproteins corrected for base protein.

Table 1Descriptions of 108 Antibodies Used in Our RPPA Studies and Their Dilutions^a

antibody target	catalog	source	specie	first Ab dilution	second Ab dilution	batch
14-3-3 ϵ	sc-23957	Santa Cruz	Mouse	1:500	1:15000	1
β -Actin	A5441	Sigma	Mouse	1:2000	1:15000	1
β -Actin	A5441	Sigma	Mouse	1:2000	1:15000	2
ADOMET	Lab	David Feith	Rabbit	1:500	1:15000	2
AIF	sc-13116	Santa Cruz	Mouse	1:500	1:15000	1
Akt	9272	Cell Signaling	Rabbit	1:250	1:15000	1
Akt(Thr308)	9275	Cell Signaling	Rabbit	1:250	1:15000	1
Akt(Ser473)	9271	Cell Signaling	Rabbit	1:150	1:15000	2
AMPK α	2532	Cell Signaling	Rabbit	1:200	1:15000	2
AMPK α (Thr172)	2535	Cell Signaling	Rabbit	1:200	1:15000	2
Antizyme inhibitor	Lab	David Feith	Rabbit	1:500	1:15000	2
Bad	9292	Cell Signaling	Rabbit	1:100	1:15000	2
Bad(Ser136)	9295	Cell Signaling	Rabbit	1:50	1:15000	2
Bad(Ser155)	9297	Cell Signaling	Rabbit	1:100	1:15000	2
Bax	2772	Cell Signaling	Rabbit	1:100	1:15000	2
Bcl2	M0887	Dako	Mouse	1:200	1:15000	2
Bcl-xL	2762	Cell Signaling	Rabbit	1:200	1:15000	2
BID	2002	Cell Signaling	Rabbit	1:500	1:15000	1
Bim	1036-1	Epitomics	Rat	1:200	1:15000	2
Cleaved Caspase-3(Asp175)	9664	Cell Signaling	Rabbit	1:250	1:15000	1
Cleaved Caspase-7(Asp198)	9491	Cell Signaling	Rabbit	1:250	1:15000	1
Cleaved Caspase-9(Asp315)	9505	Cell Signaling	Rabbit	1:250	1:15000	1
Cleaved Caspase-9(Asp330)	9501	Cell Signaling	Rabbit	1:250	1:15000	1
Caspase-3	9662	Cell Signaling	Rabbit	1:500	1:15000	1
Caspase-8	9746	Cell Signaling	Mouse	1:250	1:15000	1
β -Catenin	9562	Cell Signaling	Rabbit	1:250	1:15000	2
β -Catenin(Ser33/37/Thr41)	9561	Cell Signaling	Rabbit	1:500	1:15000	2
CD11a(Integrin α^L)	610826	Bd Transduction Lab	Mouse	1:500	1:15000	2

antibody target	catalog	source	specie	first Ab dilution	second Ab dilution	batch
CD49b(Integrin α_2)	611016	Bd Transduction Lab	Mouse	1:500	1:15000	2
CDC2	CC01	Calbiochem	Mouse	1:200	1:15000	2
CDK2	SC-6248	Santa Cruz	Mouse	1:200	1:15000	2
CDK4	2906	Cell Signaling	Mouse	1:200	1:15000	2
cIAP-1	07-759	Upstate	Rabbit	1:200	1:15000	1
Cyclin B1	SC-245	Santa Cruz	Rabbit	1:200	1:15000	2
Cyclin D1	sc-718	Santa Cruz	Rabbit	1:1000	1:15000	1
Cyclin E	sc-247	Santa Cruz	Mouse	1:250	1:15000	1
EGFR	sc-03	Santa Cruz	Rabbit	1:500	1:15000	1
EGFR(Tyr992)	2235	Cell Signaling	Rabbit	1:50	1:15000	2
Elk-1(Ser383)	9181	Cell Signaling	Rabbit	1:100	1:15000	1
Erg-1/2/3	sc-353	Santa Cruz	Rabbit	1:500	1:15000	1
Erk 2	sc-154	Santa Cruz	Rabbit	1:1000	1:15000	1
Erk1/2(Thr202/Tyr204)	9101	Cell Signaling	Rabbit	1:1000	1:15000	1
FoxO3a	9467	Cell Signaling	Rabbit	1:500	1:15000	1
FoxO3a(Ser318/321)	9465	Cell Signaling	Rabbit	1:500	1:15000	1
Gab2	3239	Cell Signaling	Rabbit	1:500	1:15000	2
Gab2(Tyr452)	3882	Cell Signaling	Rabbit	1:250	1:15000	2
GSK-3	9278	Cell Signaling	Rabbit	1:200	1:15000	1
GSK-3 α /pS _{er} 21/9)	9331	Cell Signaling	Rabbit	1:200	1:15000	1
HDAC 3	2632	Cell Signaling	Rabbit	1:100	1:15000	2
HER2/erbB2	2242	Cell Signaling	Rabbit	1:500	1:15000	2
HER2/erbB2(Tyr1248)	06-229	Upstate	Rabbit	1:1000	1:15000	2
HER3/erbB3	05-390	Upstate	Mouse	1:500	1:15000	2
HOXA10	AVARP13071	Aviva systems	Rabbit	1:500	1:15000	2
HSP90	4875	Cell Signaling	Rabbit	1:500	1:15000	2
JAB1	sc-13157	Santa Cruz	Mouse	1:500	1:15000	2
JNK1/3	sc-474	Santa Cruz	Rabbit	1:200	1:15000	1
JunB	3755	Cell Signaling	Rabbit	1:500	1:15000	2
LKB1	3050	Cell Signaling	Rabbit	1:500	1:15000	2
MDM2	sc813	Santa Cruz	Rabbit	1:1000	1:15000	2

antibody target	catalog	source	specie	first Ab dilution	second Ab dilution	batch
MEK1/2	9122	Cell Signaling	Rabbit	1:2000	1:20000	1
MEK1/2(Ser217/221)	9121	Cell Signaling	Rabbit	1:2000	1:20000	1
c-Met(Tyr1230/Tyr1234/Tyr1235)	44-888G	Biosource	Rabbit	1:50	1:15000	1
MSI2	MAB10085	Chemicon	Mouse	1:500	1:15000	2
mTOR(Ser2448)	2971	Cell Signaling	Rabbit	1:250	1:15000	1
c-Myc	9402	Cell Signaling	Rabbit	1:200	1:15000	1
NF-kB(Ser276)	3034	Cell Signaling	Rabbit	1:1000	1:15000	1
NPM	3542	Cell Signaling	Rabbit	1000	1:15000	2
Nur77	IMG-528	Imgenex	Rabbit	1:200	1:15000	
ODC	Lab	Lisa Shantz	Rabbit	1:100	1:15000	1
p16	sc468	Santa Cruz	Rabbit	1:2000	1:15000	2
p21	2946	Cell Signaling	Mouse	1:250	1:15000	2
p27	SC-528	Santa Cruz	Rabbit	1:250	1:15000	2
p38	9212	Cell Signaling	Rabbit	1:500	1:15000	2
p38 MAPK(Thr180/Tyr182)	9211	Cell Signaling	Rabbit	1:100	1:15000	1
p53	554294	BD Pharmingen	Mouse	1:250	1:15000	2
p70 S6 Kinase(Thr389)	9205	Cell Signaling	Rabbit	1:1000	1:15000	1
PARP	9542	Cell Signaling	Rabbit	1:200	1:15000	1
Cleaved PARP(Asp214)	9546	Cell Signaling	Mouse	1:100	1:15000	1
PKC1(Ser241)	3061	Cell Signaling	Rabbit	1:500	1:15000	2
PI-3 kinase	1683-1	Epitomics	Rabbit	1:200	1:15000	2
PKC α	05-154	Upstate	Mouse	1:2000	1:15000	2
PKC α (Ser657)	06-822	Upstate	Rabbit	1:500	1:15000	2
PKC β	sc-8049	Santa Cruz	Mouse	1:300	1:15000	2
PKC δ	sc937	Santa Cruz	Rabbit	1:200	1:15000	1
PKC δ (Ser645)	07-874	Upstate	Rabbit	1:1000	1:15000	1
PPAR γ	sc7273	Santa Cruz	Mouse	1:75	1:15000	2
PTEN	9552	Cell Signaling	Rabbit	1:1000	1:15000	2
Rac1/2/3	2465	Cell Signaling	Rabbit	1:500	1:15000	2
Raf-B	sc5284	Santa Cruz	Mouse	1:200	1:15000	2
Rb	554136	BD Pharmingen	Mouse	1:250	1:15000	1

antibody target	catalog	source	specie	first Ab dilution	second Ab dilution	batch
SHIP1	sc-8425	Santa Cruz	Mouse	1:100	1:15000	1
SHIP2	2730	Cell Signaling	Rabbit	1:1000	1:15000	2
SIRT1	ab32441	Abcam	Rabbit	1:1000	1:15000	2
Smad1	1649-1	Epitomic	Rabbit	1:100	1:15000	2
Smad4	sc7966	Santa Cruz	Mouse	1:100	1:15000	2
SPMS	Lab	David Feith	Rabbit	1:500	1:15000	2
c-Src	44-656G	Biosource	Rabbit	1:500	1:15000	1
c-Src(Tyr527)	2105	Cell Signaling	Rabbit	1:1000	1:15000	1
SSAT	Lab	David Feith	Rabbit	1:500	1:15000	1
Stat1	9217	Cell Signaling	Rabbit	1:250	1:15000	1
Stat3	06-596	Upstate	Rabbit	1:1000	1:15000	1
Stat3(Tyr705)	9131	Cell Signaling	Mouse	1:1000	1:15000	1
Stat5	9352	Cell Signaling	Rabbit	1:250	1:15000	1
Survivin	2802	Cell Signaling	Mouse	1:1000	1:20000	2
TIF1	NB100-2597	Novus	Rabbit	1:2000	1:15000	2
YAP	4912	Cell Signaling	Rabbit	1:100	1:15000	2
YAP(Ser127)	4911	Cell Signaling	Rabbit	1:200	1:15000	2
ZNF342	ab51265	Abcam	Mouse	1:2000	1:15000	2

^a Phosphorylation sites are in parentheses.

Table 2

Starved-Fed Differences in Protein and Phosphoprotein Expression Detected by Antibodies^a

protein	LNZ308	SNB19	U87	U251	LN229	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MCF7
14-3-3 ϵ	1	1	0	0	0	0	-1	0	0	0	1	0
ADOMET	1	1	1	0	0	-1	0	0	0	1	-1	-1
AIF	1	1	0	0	-1	0	1	0	1	1	0	-1
Akt 1	1	0	0	-1	0	0	1	-1	-1	-1	-1	-1
Akt(Ser473)	1	-1	1	1	-1	1	-1	0	0	-1	0	-1
Akt(Thr308)	1	-1	-1	1	1	0	-1	-1	0	-1	0	0
AMPK α	0	0	0	1	0	0	0	1	0	-1	-1	-1
AMPK α (Thr172)	1	1	1	0	-1	-1	0	1	-1	0	0	0
Antizyme inhibitor	-1	1	1	1	0	1	1	0	-1	0	-1	0
Bad	1	0	0	0	0	0	0	1	0	0	-1	-1
Bad(Ser136)	0	0	1	0	0	-1	-1	-1	0	0	-1	-1
Bad(Ser155)	1	0	0	1	0	0	-1	0	-1	-1	0	-1
Bax	1	1	1	-1	1	0	1	-1	0	0	0	0
Bcl2	1	1	1	-1	1	-1	1	1	-1	0	-1	1
Bcl-xL	1	1	0	1	0	1	0	-1	-1	-1	-1	-1
BID	0	1	0	1	0	0	0	0	0	0	-1	-1
Bim	0	1	1	0	0	0	0	1	-1	0	0	-1
Cleaved Caspase-3(Asp175)	1	1	-1	0	0	0	1	0	0	0	0	1
Cleaved Caspase-7(Asp198)	1	1	0	0	1	0	0	1	0	0	1	0
Cleaved Caspase-9(Asp315)	1	0	0	0	0	0	0	0	0	0	1	0
Cleaved Caspase-9(Asp330)	1	0	0	0	0	-1	0	0	0	-1	0	-1
Caspase-3	1	1	0	-1	1	0	0	0	1	0	0	-1
Caspase-8	1	1	0	-1	0	1	0	0	-1	0	-1	0
β -Catenin	0	0	0	0	0	0	0	-1	-1	-1	-1	1
β -Catenin(Ser33/37/Thr41)	0	0	-1	0	-1	-1	0	0	-1	-1	-1	-1
CD11a(Integrin α ^L)	1	0	1	0	0	0	1	-1	0	0	-1	-1
CD49b(Integrin α ₂)	1	1	1	0	1	-1	0	0	-1	1	-1	1
CDC2	0	1	1	0	0	1	-1	0	-1	-1	-1	-1

protein	LNZ308	SNB19	U87	U251	LN229	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MCF7
CDK2	1	0	1	0	0	-1	0	-1	-1	0	-1	0
CDK4	0	1	1	-1	1	1	-1	1	0	0	-1	-1
cIAP-1	0	1	0	1	-1	0	0	-1	0	-1	-1	-1
Cyclin B1	1	1	1	0	0	1	0	-1	0	0	0	-1
Cyclin D1	0	0	0	0	-1	-1	0	-1	0	0	0	-1
Cyclin E	1	1	0	0	0	-1	0	0	0	0	0	0
EGFR	1	0	0	1	-1	1	0	0	0	-1	-1	-1
EGFR(Tyr992)	1	0	1	0	-1	0	0	0	0	1	-1	-1
Elk-1(Ser383)	0	0	0	0	0	0	-1	0	1	-1	-1	-1
Erg-1/2/3	1	0	1	0	1	0	0	0	-1	0	-1	0
Erk 2	1	1	0	0	1	1	0	0	1	0	-1	0
Erk1/2(Thr202/Tyr204)	0	1	1	-1	1	-1	1	1	-1	0	1	-1
FoxO3a	1	0	1	0	0	1	-1	0	-1	0	0	-1
FoxO3a(Ser318/321)	0	1	1	1	0	1	-1	1	0	0	0	0
Gab2	1	-1	-1	0	1	0	-1	1	0	-1	-1	-1
Gab2(Tyr452)	0	0	1	0	0	1	-1	0	-1	0	0	-1
GSK-3	1	1	0	0	0	0	0	-1	0	-1	0	-1
GSK-3 α /Ser21/9	0	-1	-1	-1	1	-1	0	-1	0	-1	0	1
HDAC 3	1	1	0	1	0	-1	-1	0	-1	0	-1	-1
HER2/ERBB2	-1	1	0	0	1	0	0	1	0	0	-1	-1
HER2/ERBB2(Tyr1248)	1	1	-1	1	-1	0	1	0	-1	0	0	-1
HER3/ERBB3	1	0	1	0	1	0	1	0	-1	0	0	-1
HOXA10	0	0	0	0	-1	0	1	-1	-1	-1	-1	0
HSP90	0	1	0	1	0	0	0	0	1	-1	0	1
Jab1	1	1	1	0	0	0	-1	-1	0	0	0	0
Jnk	1	0	0	1	0	0	-1	0	0	0	1	0
JunB	0	1	0	0	0	-1	0	0	0	0	-1	-1
LKB1	1	1	0	0	0	0	0	0	1	0	-1	1
MDM2	1	1	1	0	0	0	0	0	-1	0	-1	-1
MEK1/2	1	1	0	1	0	0	0	-1	0	0	-1	-1
MEK1/2(Ser217/221)	0	0	0	-1	1	-1	0	0	-1	-1	1	-1

protein	LNZ308	SNB19	U87	U251	LN229	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MCF7
c-Met(Tyr1230/Tyr1234/Tyr1235)	1	0	0	0	-1	0	0	-1	0	0	1	0
MSI2	1	1	1	1	-1	0	0	-1	0	0	-1	0
mTOR(Ser2448)	1	0	0	0	1	0	0	0	0	0	1	0
c-Myc	0	0	-1	0	0	-1	-1	0	-1	-1	-1	0
NF- κ B p65(Ser276)	1	1	0	-1	1	0	0	0	0	0	0	-1
NPM	0	0	-1	1	0	1	1	0	1	-1	-1	0
Nur77	1	0	1	1	0	0	0	-1	0	0	0	-1
ODC	0	0	0	1	1	0	0	0	0	-1	0	-1
p16	1	1	1	1	-1	1	1	0	0	-1	0	-1
p21	0	1	1	-1	1	0	0	1	-1	1	0	0
p27	1	0	0	1	0	-1	0	1	-1	0	-1	0
p38	0	0	1	0	0	-1	0	0	0	0	-1	-1
p38 MAPK(Thr180/Tyr182)	1	0	-1	0	-1	-1	0	0	0	-1	1	-1
p53	-1	-1	1	1	0	1	0	1	1	-1	1	1
p70 S6 Kinase(Thr389)	0	-1	-1	0	-1	0	0	0	-1	-1	1	1
PARP	1	1	0	-1	0	0	0	0	0	0	0	-1
Cleaved PARP(Asp214)	0	1	-1	0	0	0	1	-1	1	0	1	-1
PDK1(Ser241)	0	0	0	1	0	1	0	0	0	0	0	0
Pl-3 kinase	1	1	1	0	0	0	0	-1	0	0	-1	-1
PKC α	0	0	0	-1	1	1	1	1	-1	-1	0	1
PKC α (Ser657)	1	1	1	1	-1	0	0	1	-1	-1	-1	1
PKC β	1	1	0	1	-1	-1	0	-1	0	-1	-1	-1
PKC δ	0	0	1	0	0	0	0	0	-1	1	0	0
PKC δ (Ser645)	1	0	-1	0	0	0	0	-1	-1	-1	0	0
PPAR γ	0	0	1	0	0	1	0	-1	0	0	-1	-1
PTEN	0	1	0	-1	1	-1	1	1	1	1	0	0
Rac1/2/3	1	1	1	0	0	0	0	0	0	0	0	-1
Raf-B	0	0	1	0	1	0	0	1	0	0	0	-1
Rb	0	-1	-1	0	1	0	0	0	0	0	-1	1
SHIP1	1	0	-1	0	-1	0	-1	0	0	-1	1	-1
SHIP2	0	0	1	0	1	0	0	1	0	0	-1	0

protein	LNZ308	SNB19	U87	U251	LN229	MDA231	KM125c	OVCAR5	MiaPaCa	MDA468	SKOV3	MCF7
Sirt1	0	0	1	1	0	0	-1	0	1	0	-1	0
Smad1	0	0	0	0	0	0	0	0	0	-1	0	-1
Smad4	0	-1	-1	0	1	-1	0	1	1	0	-1	-1
SPMS	1	1	0	1	0	-1	0	-1	0	0	-1	0
c-Src	0	0	0	1	-1	0	0	0	0	-1	-1	0
c-Src(Tyr527)	1	0	0	1	-1	0	0	0	0	-1	1	-1
SSAT	1	1	0	0	0	1	1	0	0	1	1	-1
Stat1	1	1	1	0	-1	0	0	0	0	0	0	1
Stat3	1	1	1	0	-1	0	0	0	0	0	0	0
Stat3(Tyr705)	1	0	1	0	0	1	-1	0	1	0	-1	-1
Stat5	1	0	-1	-1	-1	0	0	-1	1	0	1	-1
Survivin	1	0	0	0	1	0	0	-1	0	-1	-1	-1
TTF1	1	-1	0	0	0	1	0	1	-1	0	-1	0
YAP	0	0	1	1	0	0	-1	0	-1	0	-1	-1
YAP(Ser127)	0	0	0	1	-1	0	0	-1	0	-1	-1	0
ZNF342	1	1	1	0	0	0	0	-1	0	0	0	0
Sum Score	60	40	26	16	2	-2	-3	-8	-19	-28	-35	-45

^aScores of -1, 0, or +1 were assigned depending on the quartile distribution of raw antibody difference values, where -1 denotes differences in the lowest quartile, +1 those in the highest quartile, and 0 all others.