Pathway-specific analysis of gene expression data identifies the PI3K/Akt pathway as a novel therapeutic target in cervical cancer

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**Statement of Translational Relevance:**

Metabolic response (as determined by post-therapy FDG-PET scanning) is predictive of survival outcome after chemoradiation in cervical cancer. The objective of this study was to use gene expression profiling as a discovery tool to identify signaling pathways associated with cervical tumor metabolic response. Using gene set enrichment analysis (GSEA), we identified alterations in expression of genes from the PI3K/Akt pathway (n=62) that were associated with incomplete metabolic response to chemoradiation. These results were validated using immunohistochemistry for pAkt and a pretreatment tumor biopsy microarray (n=174). Pretreatment phosphorylation of Akt was common in our dataset (88%), and overexpression of pAkt was associated with decreased survival outcome after radiotherapy. Expression of pAkt was associated with increased pretreatment FDG uptake in cervical tumors *in vivo* and inhibition of PI3K decreased FDG uptake *in vitro*. These results suggest that targeted inhibition of the PI3K/Akt pathway may improve treatment outcome in cervical cancer.

**Abstract:**

**Purpose**

Cervical tumor response on post-therapy ¹⁸F-fluorodeoxyglucose positron emission tomography (FDG-PET) is predictive of survival outcome. The purpose of this study was to use gene expression profiling to identify pathways associated with tumor metabolic response.

**Experimental Design**
This was a prospective tissue collection study for gene expression profiling of 62 pretreatment biopsies from patients with advanced cervical cancer. Patients were treated with definitive radiation. Fifty-three patients received concurrent chemotherapy. All patients underwent a pretreatment and a 3-month post therapy FDG-PET/CT. Tumor RNA was harvested from fresh frozen tissue and hybridized to Affymetrix U133Plus2 GeneChips. Gene set enrichment analysis (GSEA) was used to identify signaling pathways associated with tumor metabolic response. Immunohistochemistry and in vitro FDG uptake assays were used to confirm our results.

**Results**

There were 40 biopsies from patients with a complete metabolic response (PET negative group) and 22 biopsies from patients with incomplete metabolic response (PET positive group). The 3-year cause-specific survival estimates were 98% for the PET negative group and 39% for the PET positive group (p<0.0001). GSEA identified alterations in expression of genes associated with the PI3K/Akt signaling pathway in patients with a positive follow up PET. Immunohistochemistry using a tissue microarray of 174 pretreatment biopsies confirmed pAkt as a biomarker for poor prognosis in cervical cancer. The PI3K inhibitor LY294002 inhibited FDG uptake in vitro in cervical cancer cell lines.

**Conclusions**

Activation of the PI3K/Akt pathway is associated with incomplete metabolic response in cervical cancer. Targeted inhibition of PI3K/Akt may improve response to chemoradiation.
Introduction:

Cervical cancer ranks among the top three cancer diagnoses in women worldwide and is a leading cause of cancer death in developing countries. In the US in 2011, 12,710 new diagnoses and 4,290 cancer deaths are expected.(1) Patients who present with locally advanced carcinoma of the cervix are treated with definitive chemoradiation therapy. Most commonly, single agent cisplatin is given once weekly for six cycles concurrently with radiation. Expected 5 year overall survival for patients with locally advanced cervical carcinoma treated in this manner is 70-80%.(2, 3)

Therapeutic response as determined by post-therapy FDG-PET and more recently FDG-PET/CT, has been shown to be predictive of progression-free and overall survival outcomes.(4-6) In a prospective data collection study at our institution, 3-year cause-specific survival was 100% and 51% for patients with a complete versus a partial metabolic response on 3 month post therapy FDG-PET (p<0.001). Corresponding 3-year progression-free survivals were 78% and 35% (p<0.0001), respectively. Multivariate analysis demonstrated that metabolic response was more predictive of treatment outcome than all known pretreatment related factors, including FIGO stage and lymph node status. Post-therapy FDG-PET may therefore be used as an immediately available surrogate biomarker for overall response to therapy.

Microarray analysis of tissue biopsy specimens has been widely implemented as a high throughput method for the detection of altered gene expression. With respect to cervical carcinoma, gene expression profiling has been used in several small studies to identify genes associated with poor outcome after treatment.(7-11) More recently, Lando et al analyzed gene dosage alterations in 97 cervical cancer patients using array comparative genomic hybridization (aCGH).(12) Their analysis identified losses in 3 chromosomal regions (3p, 13q and 21q) that were associated with poor outcome after
chemoradiotherapy in cervical cancer. Integration of the aCGH data with gene expression data identified 4 candidate genes associated with poor prognosis after chemoradiation treatment (RYBP, GBE1, FAM48A, MED4). Gene ontology analysis of their results implicated several biologic processes in chemoradiation response in cervical cancer including protein translation, carbohydrate metabolism, apoptosis and chromosomal maintenance.

Very few studies have explored the molecular mechanisms that regulate treatment response in cervical cancer, and no studies have specifically examined the signaling pathways that influence cervical tumor metabolic response. The objective of the current study was to use gene expression profiling as a discovery tool to identify signaling pathways associated with incomplete metabolic response after chemoradiation therapy in cervical cancer.

**Materials and Methods:**

**Patients**

The study population included 62 patients prospectively enrolled into a tumor banking protocol at the time of diagnosis of cervical cancer (March 1998 through December 2006). Eligibility criteria for tumor banking were: 1) patients had a pathologic diagnosis of invasive carcinoma of the cervix; 2) patient age was greater than or equal to 18 years; 3) patient’s tumor had adequate tissue to obtain a 1 cm biopsy and 4) patients were scheduled to undergo radiation therapy with or without chemotherapy in the Department of Radiation Oncology at Washington University School of Medicine. As part of the tumor banking protocol, tumor biopsies were obtained and frozen prior to the initiation of therapy. As part of standard practice at our institution, both pretreatment
and post-treatment FDG-PET/CT imaging studies were obtained. Approval from the institutional Human Research Protection Office was obtained for this study and all patients signed informed consent.

Over the time period 1998-2006, 131 patients contributed specimens to the tumor bank. After pathologic review, 86 specimens were found to have >25% tumor content and minimal contaminating normal cells (blood, lymphocytes or cervical stromal cells). Gene chip hybridization was performed on 70 specimens with the sufficient RNA yield and quality for hybridization. Prior to data analysis, 8 samples were excluded due to additional data from pathologic review (3), absence of 3 month follow up PET data (3) or duplicate biopsies from the same patient (2). Patient samples were divided into 2 data sets for GeneChip hybridization and data analysis (see GeneExpression Profiling below). Clinical characteristics between the 2 groups were similar (Table 1). The test and validation sets consisted of 20 and 42 pretreatment cervical biopsies, respectively. Radiotherapy was performed in all patients and consisted of external irradiation and intracavitary brachytherapy. Concurrent chemotherapy (weekly cycles of 40 mg/m² cisplatin) was administered to 53 patients.

**Clinical Follow Up**

Clinical follow up was performed 6 weeks after the completion of therapy. Post-therapy FDG-PET was performed 3 months after the completion of therapy. Patients were followed clinically thereafter according to institutional guidelines. Median follow up time for patients alive at the time of last follow up was 68 months (range 15-134 months). At the time of last follow up, 3 patients were alive with cervical cancer and 18 patients had died due to cervical cancer. Three patients died due to intercurrent illness,
and 2 patients died due to toxicity. The remaining 36 patients had no evidence of disease.

**PET Imaging**

Before November 2002, FDG-PET was performed using a conventional PET scanner and interpreted as previously described.\(^{(13)}\) Thereafter, all FDG-PET studies were performed with a hybrid PET/CT scanner using methods described by Wright et al.\(^{(14)}\) For patients in this study, 24 were imaged with conventional PET and 38 were imaged with PET/CT. PET studies were deferred until the blood glucose concentration was less than 200 mg/dL. PET/CT images were interpreted in a standard clinical fashion, both separately and in a fused mode. Standardized uptake value (SUV\(_{\text{max}}\)) was determined for primary tumors from the pretreatment FDG-PET as previously described.\(^{(15)}\) Three month follow up PET results were reviewed and compared to the pretreatment PET study as previously described.\(^{(6)}\) There were 40 pretreatment biopsies from patients with no residual FDG uptake on the 3 month post-therapy PET (PET negative group). The PET positive group consisted of 22 pretreatment biopsies from patients with residual or progressive disease on the 3 month post therapy PET. Within the PET positive group, there were 14 pretreatment biopsies from patients with persistent FDG uptake on the 3 month post therapy PET in sites of abnormal FDG uptake on the pretreatment study (partial metabolic response) and 8 pretreatment biopsies from patients with new sites of abnormal FDG uptake on the 3 month post therapy PET (progressive disease).

**Statistical Analysis**
Survival and tumor recurrence were measured from the completion of treatment. The Kaplan-Meier (product-limit) method was used to derive estimates of survival, based on total sample size. StatView version 5.0.1 software (SAS Institute, Cary, NC) was used for the analysis. P< .05 was set as the threshold for significance for all study outcomes. Tests of equivalence of estimates of survival were performed by the generalized Wilcoxon log-rank test. A paired t-test was used to compare the results of pAkt staining to pretreatment cervix tumor standardized uptake value (SUV$_{\text{max}}$).

**Gene Expression Profiling**

Pretreatment tumor biopsies were frozen at the time of collection. Frozen sections were histologically reviewed for documentation of invasive cancer; only biopsies with > 25% tumor were included in this study. Tumor RNA was harvested from fresh frozen tissue using Trizol reagent (Invitrogen, Carlsbad, CA) as described.(16) RNA samples were then labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 expression microarrays (Affymetrix, Santa Clara, CA) using standard protocols from the Laboratory for Clinical Genomics.(16, 17) To perform inter-array comparisons, the raw scan data from each microarray were scaled to a target intensity of 1500 using the Affymetrix GCOS 1.2 (MAS 5) statistical algorithm (http://www.affymetrix.com).

Basic microarray data visualization, data filtering and hierarchical clustering were performed using the Spotfire DecisionSite for Functional Genomics (Somerville, MA) as described previously.(16) Gene set enrichment analysis (GSEA) (http://www.broad.mit.edu/gsea) identified signaling pathways associated with tumor metabolic response. Depending on sample size, phenotype or geneset permutation analysis with ratio-of-classes or signal-to-noise gene ranking was performed, as recommended by the program authors.
Immunohistochemistry

To generate a validation set for our gene expression data, a tissue microarray (TMA) was constructed from 174 archived paraffin-embedded pretreatment cervical cancer biopsies. Approval for construction of the tissue microarray using archived specimens was obtained from the Washington University Human Research Protection Office. A waiver of informed consent was obtained. Briefly, slides were reviewed by a gynecologic pathology specialist (PCH). The tumors were histologically typed as squamous cell carcinoma (n=149), adenocarcinoma (n=10), or other (n=5). Areas containing invasive carcinoma were indicated on the glass slides by marking them with a dotting pen. Paired 1 mm punches were then taken from the corresponding regions of the paraffin blocks and placed into tissue microarray blocks. Unstained 4 µm sections from these blocks were utilized for pAkt staining. The TMA slides were dewaxed in xylene and rehydrated using graded alcohols. The slides were immersed in 100 uM citrate buffer and boiled for 10 minutes in a pressure cooker to retrieve antigen. Endogenous peroxidase was blocked by applying 0.3% hydrogen peroxide for 20 minutes. Sections were subsequently blocked in 5% goat serum in TBS-Tween-20. The rabbit monoclonal anti-human pAkt 473 antibody (736E11, Cell Signaling Technology, 1:50) was used as a primary antibody overnight at 4C. Goat anti-rabbit (sc2040, Santa Cruz) was used as a secondary antibody. Slides were developed in diaminobenzidine, dehydrated and mounted. Slides were scored for pAkt intensity by two independent observers who were blinded to corresponding clinical data (0-negative; 1-weak; 2-intermediate; 3-strong). One hundred and sixty four out of 174 (93%) of the biopsies had interpretable pAkt
IHC results. The remaining 10 were uninterpretable due to artifact or detachment of the biopsy from the glass slide during processing.

**Western blotting**

Cervix cell lines SW756, Caski, C33A, C41, Me180, HeLa, HT-3 and SiHa were grown in standard media supplemented with fetal bovine serum to 70-80% confluence. Cells were washed twice with PBS and extracted in lysis buffer containing 1% NaF, 0.5% Na$_3$VO$_4$, and protease inhibitor. Equal amounts of proteins from each sample were added in loading buffer and were heated for 3 min at 100°C. Samples were loaded on 10% SDS-PAGE gel for running 2 h and were then transferred onto Bio-Rad nitrocellulose membranes (Bio-Rad, CA). After blocking for 1 h with 5% nonfat dry milk, blots were probed overnight at 4°C with primary antibodies against pAkt$^{\text{Ser473}}$ (1:1000; Cell Signaling Technology, MA), total Akt (1:1000, Cell Signaling Technology, MA), or β-Actin (1:10000, Sigma, MO). β-Actin was used as the internal control. Blots were probed with HRP-conjugated anti-rabbit or anti-mouse polyclonal IgG secondary antibodies for 1 h at RT. After incubation in chemiluminescent substrate (ECL Western blotting detection reagents, Amersham/GE Healthcare) for 1 min, blots were exposed on Classic Film BX (MIDSCI, MO).

**FDG uptake assays**

Cervix cell lines SiHa, ME-180 and C33A were grown in media supplemented with fetal bovine serum to 70-80% confluence. Thirty minutes prior to adding radiolabeled glucose, media was changed to Glucose-free DMEM + 10% FBS. Cells were incubated for 1 hour at 37°C in each of the following conditions: 20 uCi FDG alone,
20 uCi FDG + 5mM glucose and 50 uM cytochalasin B, 20 uCi FDG and 100 uM LY294002 (Cell Signaling Technology, Danvers, MA). Cells were rinsed in cold PBS, harvested in 500 ul of 1% SDS + 10 mM Na borate and counted on a gamma counter.

**Results:**

*Metabolic Response and Survival Outcome*

There were 40 pretreatment biopsies from patients with no evidence of disease on the 3 month post-therapy PET (PET negative group) and 22 pretreatment biopsies from patients with residual or progressive disease on the 3 month post-therapy PET (PET positive group). The 3-year cause-specific survival estimates were 98% for the PET negative group and 39% for the PET positive group (Figure 1A). The corresponding 3-year progression-free survival estimates were 84% and 32%, respectively (Figure 1B).

*Gene Expression Profiling*

We examined global gene expression in cervical tumors as a means to identify signaling pathways whose up- or down-regulation correlated with tumor metabolic response, as demonstrated by 3-month post-therapy FDG-PET/CT. The patient population consisted of 62 women diagnosed with cervical cancer; see Table 1 for clinical characteristics. Tissue from tumor biopsies was collected and fresh frozen for subsequent mRNA isolation, quality assessment, and performance of Affymetrix U133+2 gene expression microarrays, as described previously. Because tumors were banked and subjected to microarray analysis in 2 discrete timeframes, during which interim there was a change in the Affymetrix RNA target preparation method, the data was divided into 2 groups, a training (20 tumors) and a test or validation set (42
tumors) based on these timeframes. Clinical characteristics were similar between the 2
groups (Table 1).

The specific goal of the gene expression analysis was the identification of
dysregulated signaling pathways associated with tumor metabolic response. Gene Set
Enrichment Analysis (GSEA) (http://www.broad.mit.edu/gsea), a powerful bioinformatic
tool that assesses whether an a priori defined set of genes (e.g., those in a common
signaling pathway) shows statistically significant, concordant differences between two
biological states (i.e. phenotypes). Therefore, we used GSEA to test whether the
expression of known oncogenic or metabolic pathways in cervical tumors was
significantly correlated with metabolic response measured by 3-month PET results. A
literature search was performed to identify signaling pathways previously implicated in
therapeutic response for cervical cancer. The results of this literature search identified
Ras pathway, Hypoxia/HIF1/VEGF, NFkB/immune modulators, PI3K/PTEN/Akt/mTOR,
and EGFR as possible targets. In addition, all available pathways related to glucose
metabolism were tested (GLUTs/glycolysis/glucose metabolism). All pathways analyzed
demonstrated a subset of upregulated genes in PET positive tumors compared to PET
negative tumors. However, overlapping pathways involving PI3K, AKT, and PTEN
signaling demonstrated strikingly significant upregulation of the entire pathway (FDR q-
values <0.05). These expression differences are exemplified by the KEGG endometrial
cancer signaling pathway (http://www.genome.jp/kegg/pathway/hsa/hsa05213.html),
which includes PI3K, AKT, and PTEN signaling. This pathway demonstrated significant
enrichment in PET positive versus PET negative tumors (FDR q-value = 0.006) (Figure
2). Genes with higher expression in PET positive tumors have higher enrichment scores
(ES) and are plotted on the left portion of the graph, while those with lower expression
in PET positive tumors have lower ES and are plotted on the right portion of the graph.
The net effect is a sigmoidal curve. The bottom portion of the plot shows the value of the ranking metric moving down the list of ranked genes. A positive ranking metric indicates that a gene is correlated with the PET positive phenotype. The table in Figure 2 enumerates the genes in the pathway for which a majority of probesets were significantly enriched and upregulated in PET positive versus PET negative tumors. Note that members of the AKT, PI3K, and PTEN pathways are significantly enriched.

**Immunohistochemistry**

To test whether Akt pathway activation is associated with survival outcome in human cervical cancers at the protein level, we performed pAkt immunohistochemistry using a commercially available anti-pS473 antibody and a tissue microarray of 174 archived paraffin embedded pretreatment cervical cancer biopsies. Two independent observers blinded to the clinical outcome and metabolic response data interpreted the results. The majority of the biopsies (88%) demonstrated some pAkt signal. Within this group, variation in intensity of pAkt staining was observed: 68% had weak staining, 26% medium staining and 6% strong staining (Figure 3).

The results of pAkt immunohistochemistry were compared to clinical outcome and risk factors for recurrence, including both pre- and post-treatment FDG-PET scan results. A paired t-test was performed to test the association between pAkt staining and FDG uptake on the pretreatment FDG-PET (as assessed by maximum standardized uptake value/SUV\textsubscript{max}). The results showed a statistically significant association between pAkt staining intensity and increased SUV\textsubscript{max} on
the pretreatment PET, implying that activation of Akt in cervical tumors is associated with increased glucose uptake \textit{in vivo} (mean difference -10.9, p < 0.0001, DF 113).

There was an association with high pAkt signal by IHC and decreased progression free survival in patients with squamous cell carcinoma (Figure 4). Five out of six (83%) patients with high pAkt expression have had a recurrence with a mean time to recurrence of 12.8 months. In the low/no pAkt expression group, 64 out of 143 (45%) patients have had a recurrence with a mean time to recurrence of 16.1 months.

\textit{Glucose uptake assays}

To test whether the PI3k/Akt pathway regulates glucose uptake in cervical cancer cells, we began by performing western blots for pAkt using 8 different cervical cancer cell lines. These results show a range of baseline pAkt expression in cervical cancer cell lines (Figure 5a), with C33A and ME180 cells having relative high baseline expression of pAkt versus SiHa cells which had little to no baseline expression of pAkt. We hypothesized that inhibition of the PI3K/Akt pathway may affect glucose uptake and this was tested by performing in vitro FDG uptake assays in the presence and absence of the PI3K inhibitor, LY294002. These results show that inhibition of PI3K decreases glucose uptake in cervical cell lines in vitro, although the effect was still present in cell lines that did not overexpress pAkt (Figure 5b).

\textit{Discussion}
Our previous clinical data demonstrated that cervical cancer patients with incomplete metabolic response after chemoradiation have poor survival outcome.\(^6\) In the current study, we used GSEA and 62 fresh frozen pretreatment cervical cancer biopsies to identify signaling pathways that are associated with post-treatment metabolic response in cervical cancer. GSEA identified reproducible alterations in expression of genes from the PI3K/Akt signaling pathway in patients with incomplete metabolic response. To confirm these results at the protein level, we tested 174 archived paraffin embedded specimens for pAkt expression using immunochemistry. Our results show that pretreatment pAkt expression is common in cervical cancer (88% pAkt positive). In the subset of patients with squamous cell histology, increased expression of pAkt was associated with decreased progression-free survival outcome after chemoradiation. In addition, increased expression of pAkt was associated with increased glucose uptake on the pretreatment FDG-PET, suggesting that pAkt expression is associated with tumor glucose uptake in vivo.

The PI3K/Akt signaling pathway is known to be dysregulated in several cancer sites, most notably endometrium where loss of PTEN and mutations of PIK3CA have been reported. For cervical cancer, a detailed analysis of the PI3K/Akt pathway using human tumor samples is lacking; however, the cumulative results of several small studies suggest that the PI3K/Akt pathway is activated in cervical cancer. In a Norwegian study of 46 paraffin embedded specimens with no associated clinical outcome data, pAkt staining was positive in 39 (85%) of the samples.\(^{18}\) In a Korean study of 27 patients (9 with recurrence and 18 without), expression of pAkt was associated with local recurrence after primary radiotherapy.\(^{19}\) Our immunohistochemistry results with 174 patient samples are consistent with these
preliminary studies and validate an association between pretreatment pAkt expression and decreased survival outcome after standard chemoradiation.

Previous studies have shown that amplification of the long arm of chromosome 3 is an early event in cervical cancer tumorigenesis, a region which includes the PIK3CA gene (3q26.3). In the Norwegian study, PI3KCA gene copy number was determined by quantitative real time PCR and was estimated to be 3 or more in 28 out of 40 cases. A second group recently reported increased messenger RNA levels for PIK3CA in 12 cervical cancer specimens known to have amplification of 3q compared to normal cervix controls. These results are compelling and suggest that chromosomal amplification of the PIK3CA gene may be one mechanism of Akt activation in cervical cancer specimens. However, in a recent study of cervical cancers by integrated aCGH and gene expression analysis, although overexpression of several other genes in the 3q26 region was found to correlate with amplification, an association between overexpression of the PIK3CA gene and amplification of this chromosomal region was not found (H. Lyng, personal communication).

Additional mechanisms may exist for activation of Akt in cervical cancer and this warrants further study. In a recent study from MD Anderson, PIK3CA mutations were identified in 2/15 (13%) of cervical cancer patients. Experiments are ongoing in our laboratory to determine the mechanism of Akt activation in human cervical tumors.

Our results suggest that dysregulation of the PI3K/Akt pathway is associated with persistent or in some cases new metabolically active tumor after local radiotherapy. It is possible that activation of Akt is a marker of radiotherapy resistance in cervical cancer, and indeed, an association between Akt pathway activation and radiation resistance has been documented in other tumor sites. Using the PI3K inhibitor LY294002, Lee et al reported that pretreatment of cervical cancer cell lines increased...
radiation sensitivity in vitro.\textsuperscript{(26)} Additional study will be needed to determine whether up front treatment with PI3K or Akt inhibitors will radiosensitize cervical tumors in vivo.

Alternatively, or perhaps in combination, activation of Akt may be a marker of altered glucose metabolism in cervical cancer. In our study, increased expression of pAkt was associated with increased glucose uptake on the pretreatment FDG-PET, suggesting that pAkt expression is associated with tumor glucose uptake in vivo.

To follow up on this result, we tested a series of cervical cancer cell lines for pAkt expression and FDG uptake in vitro. When the PI3K inhibitor LY294002 was used, we observed a decrease in FDG uptake; however, this effect was also present in cell lines that did not overexpress pAkt in the baseline study. It should be noted that our western blots for baseline pAkt were performed in the presence of 25 mM glucose, and there are reports that Akt is activated when cells are exposed to glucose or during glucose uptake.\textsuperscript{(27, 28)} We have observed robust endogenous Akt activation in C33A compared to SiHa and ME-180 cells. We determined that LY294002, a known PI3K inhibitor had a greater effect in SiHa and ME180 than C33A cells. This observation suggests that while LY294002 inhibited Akt mediated glucose uptake in SiHa and ME-180, where the endogenous Akt levels are low, its effect in C33A is limited by pre-existing high endogenous phospho-Akt expression and may require a higher dose of LY294002 for the same effect. It is possible that in those cells with no basal Akt activation, presence of glucose may lead to Akt activation and subsequent glucose uptake and that this effect is significantly reduced when PI3K is inhibited. Experiments are ongoing in our laboratory to follow up on these results.

The limitations of this study are those of any gene expression profiling analysis, including the fact that mRNA expression change does not necessarily equate with functional changes to signaling pathways. However, we mitigated this limitation by using
an orthogonal method, namely immunohistochemistry assay of proteins in the Akt pathway, in a large validation set of tumor specimens, and found very similar results. Furthermore, we demonstrated functional evidence of the role of the Akt pathway by showing that the PI3K inhibitor LY294002 inhibited FDG uptake in cervical cancer cell lines.

In summary, we identified alterations in expression of genes from the PI3K/Akt pathway that are associated with metabolic response in cervical cancer (n=62). These results were validated using immunohistochemistry for pAkt and a tissue microarray (n=174). Pretreatment phosphorylation of Akt is common in cervical cancer specimens (88%), and overexpression of pAkt is associated with decreased survival outcome after radiotherapy. Expression of pAkt is associated with increased FDG uptake in cervical tumors in vivo. Inhibition of PI3K decreases FDG uptake in vitro.
**Figure Legends**

**Figure 1A.** Kaplan Meier curves for cause-specific survival for the 62 patient data set. There were 40 pretreatment biopsies from patients with no evidence of disease on the 3 month post-therapy PET (PET negative group) and 22 pretreatment biopsies from patients with residual or progressive disease on the 3 month post-therapy PET (PET positive group).

**Figure 1B.** Kaplan Meier curves for progression free survival for the 62 patient dataset.

**Figure 2.** GSEA enrichment plot of KEGG endometrial cancer pathway genes in PET + vs PET – tumors. Genes in the KEGG endometrial cancer signaling pathway demonstrated significant enrichment in PET + versus PET - tumors (FDR q-value = 0.006). The upper portion of the figure plots the enrichment scores for each gene, while the bottom portion of the plot shows the value of the ranking metric moving down the list of ranked genes. The table enumerates the genes in the pathway for which a majority of probesets were significantly enriched and upregulated in PET positive versus PET negative tumors.

**Figure 3.** Immunohistochemical staining of the tissue microarray (TMA) from human cervical cancers. Upper panel shows example pAKT437 staining from different cases: negative (case number: 115618), weak (115621), medium (115630) and strong (115584). Lower panel shows H & E staining.
Figure 4. Kaplan Meier curves for progression-free survival in patients with squamous cell carcinoma and high Akt expression versus low/no Akt expression. Pretreatment biopsies positive for squamous cell carcinoma were tested for pAkt by IHC as described in Figure 2. Results are shown for 149 patients with squamous cell histology and high pAkt IHC (n=6) vs. squamous cell histology and low or no Akt expression (n=143) (p=0.53).

Figure 5a. Western blot for total Akt and pAkt from 8 cervical cancer cell lines. Cervix cell lines SW756, Caski, C33A, C41, Me180, HeLa, HT-3 and SiHa were grown in standard media supplemented with fetal bovine serum to 70-80% confluence. Cells were washed and extracted in lysis buffer containing 1 % NaF, 0.5% Na3VO4, and protease inhibitor. Blots were probed with primary antibodies against pAktSer473 (1:1000; Cell Signaling Technology, MA), total Akt (1:1000, Cell Signaling Technology, MA), or β-Actin (1:10000, Sigma, MO). β-Actin was used as the internal control.

Figure 5b. Glucose uptake assays for cervical cancer cell lines. Cervix cell lines SiHa, ME-180 and C33A were grown in media supplemented with fetal bovine serum to 70-80% confluence. Thirty minutes prior to adding radiolabeled glucose, media was changed to Glucose-free DMEM + 10% FBS. Cells were incubated for 1 hour at 37C in each of the following conditions: 20 uCi FDG alone, 20 uCi FDG + 5mM glucose and 50 uM cytochalasin B, 20 uCi FDG and 100 uM LY294002 (Cell Signaling Technology, Danvers, MA). Cells were rinsed in cold PBS, harvested in 500 ul of 1% SDS + 10 mM Na borate and counted on a gamma counter.


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Figure 1A.

![Graph showing survival rates for PET-negative and PET-positive cases over time.]

Figure 1B.

![Graph showing survival rates for PET-negative and PET-positive cases over time.]

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Figure 2.
Pathway-specific analysis of gene expression data identifies the PI3K/Akt pathway as a novel therapeutic target in cervical cancer

Julie K Schwarz, Jacqueline E Payton, Ramachandran Rashmi, et al.

Clin Cancer Res  Published OnlineFirst January 10, 2012.