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Molecular Imaging of Akt Enables Early Prediction of Response to Molecular Targeted Therapy[1,2]

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Abstract

Development of noninvasive, real-time molecular imaging tools to assess responsiveness of a given therapy may be a critical component of the success of individualized therapy approach for patients. Toward this, we have previously developed and validated molecular sensors for Akt and caspase-3 activity, and in this report, we have explored the utility of these reporters in assessing the responsiveness of tumors to a combination of gemcitabine (Gem) and cetuximab (Cet) delivered in two opposite schedules. We found that human head and neck cancer (UMSCC1) xenografts responded significantly better in a schedule where cetuximab was administered after gemcitabine when compared with the schedule of cetuximab followed by gemcitabine. Wilcoxon two-sample tests suggested that the difference in tumor volumes in two schedules became significant on day 7 ($P > .05$ on day 4, and $P < .05$ on days 7 and 10), and the difference in activity of Akt in two schedules became significant on day 4 ($P < .05$ on days 4, 6, and 10). Using Akt reporter activity and cubic spline interpolation, the distinction between the two schedules could be detected 2 days before using the tumor volume, suggesting that molecular imaging of Akt may allow early prediction of therapy responsiveness. We did not observe a significant difference between the two schedules in the caspase-3 activity. In summary, this proof-of-concept study provides a basis for using molecular imaging of Akt as an early indicator of therapeutic efficacy.

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Introduction

The advances in cellular and molecular biology in the past century have made momentous progress in unraveling the molecular footprints of tumorigenesis by identifying the abnormalities in signal transduction pathways and gene alterations associated with cancer initiation and propagation [1]. Although a number of biomarkers have been identified and their roles were construed, the true impact of these advances is still not discernible in clinic. This is partly because most cancers are extremely heterogeneous; current efforts focus mainly on identifying biomarkers from cancer samples obtained by biopsy of tumors, which provide a snapshot of biomarkers at the time of sample retrieval, and fail to provide any information on the dynamic changes within the malignancy and its milieu. Further, clinical diagnosis and outcome of a therapy are still determined by the assessment of gross structural measurements obtained several months after the therapy has been administered. Therefore, the ability to predict therapeutic outcome early into treatment will not only provide a unique opportunity to make

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informed clinical decisions on the efficacy of a particular therapy leading to improvement in quality of life (by not subjecting them to a nonefficacious therapy and/or shifting them to alternate regimen) but also reduce expenses associated with nonefficacious therapies. Therefore, there is an urgent need for molecular imaging tools for key signaling intermediates [1]. In this vein, we have previously reported development and validation of molecular imaging reporter for monitoring activities of Akt kinase and caspase-3 protease [1–3]. Because both these enzymes play a critical role in cell proliferation and apoptosis, they are good candidates for investigating the potential of noninvasive imaging in predicting early response.

The Akt/PKB, a serine/threonine kinase that is frequently upregulated in a wide range of solid tumors and hematologic malignancies, is a central signaling hub where many upstream oncogenic stimuli (e.g., growth factor signaling/cytokine cascades) converge [4]. These signals are then relayed by Akt to downstream intermediates, such as NF-κB, mTOR, Forkhead, Bad, GSK-3, and MDM-2, which eventually modulate cellular processes such as proliferation, cell cycle progression, and protection from proapoptotic stimuli [5,6]. Because Akt is one of the key oncologic targets, immense efforts have gone into understanding its molecular biology and to develop novel therapeutic. Existing modalities, such as immunoblot analysis or immunocytochemistry using a phospho-Akt antibody [7], are invasive, cumbersome, and only provide a snapshot view of the kinase activity at a specific time point [5]. We recently developed a luciferase complementation–based molecular imaging reporter for monitoring Akt activity (bioluminescent Akt reporter, BAR), which allows noninvasive and real-time imaging of Akt activity both *in vitro* and *in vivo*. The high specificity and sensitivity of this reporter were demonstrated in a number of different cancer cells such as UMSCC1 (head and neck cancer), D54 (glioblastoma), H1975 (lung cancer), HCC-H827 (lung cancer), and DU145 (prostate cancer) and were validated using conventional techniques such as immunohistochemistry, immunoprecipitation, and immunoblot analysis [2] (Figures W1 and W2).

Caspase-3 also plays an important role in the activation of both intrinsic and extrinsic apoptotic cascades. We had previously developed a luciferase activity–based caspase-3 reporter [3] (see Supplementary Results). Recently, we developed and validated a second-generation caspase-3 reporter with a highly improved signal-to-noise ratio. This reporter was based on luciferase complementation and was exploited for the detection of caspase-3 activity both *in vitro* and *in vivo* [3]. In an experimental brain cancer therapeutic system using D54 xenografts, this molecular imaging reporter was used for optimizing doses of temozolomide (Temodar) in combination with radiation [3]. Determination of bioluminescence activity within D54 tumor xenografts revealed that control and irradiated animals had a minimal increase in bioluminescence activity, whereas temozolomide-treated animals had a nearly 50-fold increase. Further, animals treated with both temozolomide and radiation therapy showed more than a 150-fold increase in bioluminescence activity which paralleled active caspase-3 staining, demonstrating the high sensitivity and specificity of the reporter.

To test the utility of the Akt and caspase-3 reporters in early prediction of therapeutic outcome, we used a system where we thought that these signaling pathways may be important. We have previously shown that the schedule of gemcitabine followed by gefitinib is better than the converse schedule in inhibiting both cell cycle progression and growth factor signaling [8]. Treatment of UMSCC1 cells with gemcitabine followed by gefitinib resulted in arrest of cells in S-phase concurrent with decreased Akt activity, increased poly(ADP-ribose) polymerase cleavage, and increased apoptosis [8]. However, the schedule of gefitinib followed by gemcitabine did not alter Akt activity, and only minimal changes in poly(ADP-ribose) polymerase cleavage and apoptosis were noticed. Similar results were also seen in experiments in nude mice bearing UMSCC1 xenografts, in which there was greater tumor regression and apoptosis when animals received gemcitabine followed by gefitinib during the first week of therapy. Thus, these schedules of combination therapies with distinct outcome would provide an ideal platform to investigate the effectiveness of real-time Akt and caspase-3 reporter activity as an early predictor of therapeutic outcome.

Therefore, we decided to carry out a study to determine whether these Akt or caspase-3 reporters could predict the outcome of treatment of UMSCC1 xenografts by the combination of an epidermal growth factor receptor inhibitor and gemcitabine. We found that the schedule of gemcitabine followed by cetuximab (Gem → Cet) is superior when compared with the opposite schedule (Cet → Gem) in inhibition of Akt activity and tumor growth. We demonstrate that significant differences in tumor volume were detected on day 7 (P = .004) and day 10 (P < .0001) between the two schedules (Gem → Cet *vs* Cet → Gem). This system permitted us to test the effectiveness of our reporter systems. Using Akt reporter such differences were detected at earlier time points on day 4 (P = .05) and remained significant until day 10 (P = .04), suggesting that molecular imaging of Akt has the potential to allow early prediction of therapeutic response.

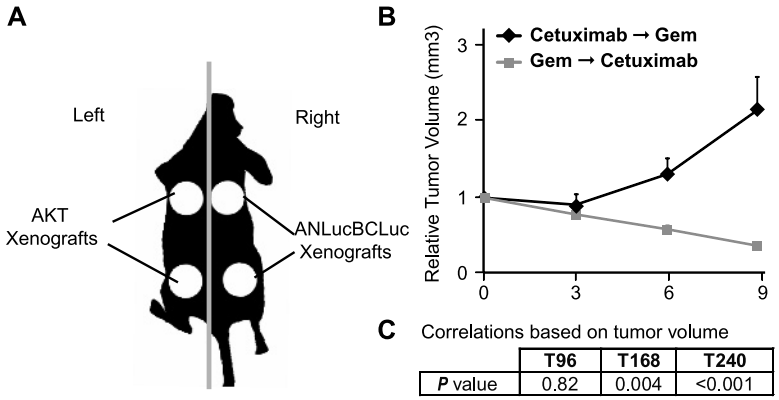
Materials and Methods

Cells Culture and In Vivo *Imaging*

UMSCC1 (human head and neck squamous carcinoma) cells were a gift from Thomas Carey, University of Michigan. UMSCC1 cells expressing BAR and caspase-3 reporter were generated as described previously [2,3,9]. Animal protocols were approved by the University Committee on the Use and Care of Animals at the University of Michigan. Before use, UMSCC1-BAR or UMSCC1-ANlucBCluc cells were trypsinized, counted, and suspended in serum-free RPMI medium for tumor implantation. Six- to eight-week-old *nu/nu* athymic male mice were maintained in ventilated cages and fed/watered *ad libitum*. Using sterile syringe and 22-gauge needle, 5×10^6 cells were then injected in a total volume of 50 μl subcutaneously at four sites as shown in Figure 1*A*. When tumor became palpable, mice were randomly divided into two groups (n = 5 per group), and then the treatment was started as shown in Figure 2, *A* and *B*. Briefly, mice were injected with either cetuximab (50 mg/kg, intraperitoneally) or gemcitabine (300 mg/kg, intraperitoneally) followed by gemcitabine or cetuximab injection next day. Bioluminescence imaging of the animals were performed as previously described [2,3,10]. Tumor volumes were recorded on days 0, 3, 6, and 9.

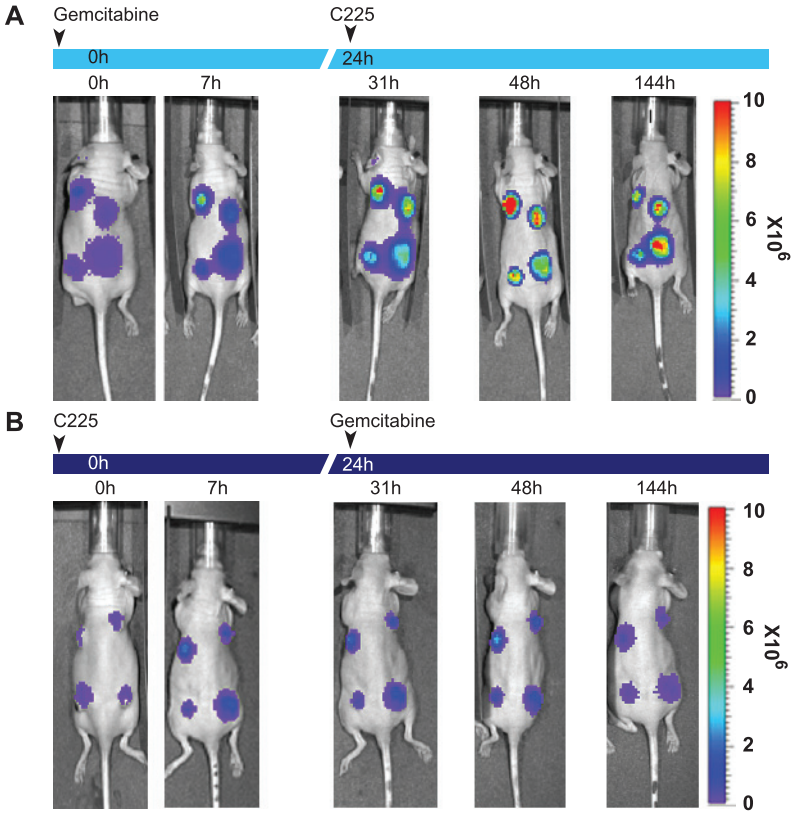
Statistical Methods

Wilcoxon two-sample test was used to compare tumor volumes, Akt and caspase-3 between two schedules (Gem → Cet *vs* Cet → Gem). Spline function was applied to the observed volume measurements (at 24, 96, 168, and 240 hours) to interpolate volumes every 24 hours until 240 hours. Similarly, Akt measurements were interpolated every 24 hours from 96 to 240 hours using the observed Akt measurements (at 25, 96, 144, and 240 hours). Wilcoxon two-sample tests were used to compare schedules at each time point (Gem → Cet *vs* Cet → Gem)



	T96	T168	T240
P value	0.82	0.004	<0.001

Figure 1. Outcome of two different schedule of gemcitabine and cetuximab combination therapy as measured by (AKT) anatomic changes. (A) Schematic representation of the UMSCC1 xenografts in nude mice where UMSCC1 cells expressing the BAR were subcutaneously implanted on the left dorsal side at both rostral and caudal regions. On the right dorsal side, caspase-3 reporter (ANlucBCluc) –bearing UMSCC1 tumor xenografts were generated. A group of five animals were subjected to one of the two schedules: Either gemcitabine followed by C225 (Gem → C225) or C225 followed by gemcitabine (C225 → Gem). (B) Tumor volumes as measured by digital calipers were plotted for each of the two schedules described above. Data are plotted as mean of 10 tumors ± SEM. (C) Wilcoxon two-sample test was used to compare tumor volumes between the two schedules (C225 → Gem *vs* Gem → C225).



C P-values based on observed Akt or caspase measurements between 2 schedules

	T4	T7	T25	T28	T31	T96	T144	T240
AKT	0.9	0.06	0.4	0.78	0.78	0.05	0.04	0.04
Caspase	0.16	0.08	1	0.48	0.74	0.29	0.12	0.18

Figure 2. Molecular imaging of two schedules of gemcitabine and cetuximab combination therapy using bioluminescence Akt and caspase-3 reporters. Nude mice were implanted with UMSCC1 xenografts expressing either BAR or ANlucBCluc reporters as demonstrated in Figure 1*A*. An increase in bioluminescence activity of Akt reporter indicates an increase in inhibition of Akt activity [2], whereas an increase in caspase-3 reporter activity is indicative of increased caspase-3 dependent apoptosis [3]. Representative overlay of bioluminescent acquisition and light photographs are shown in A for the gemcitabine-followed-by-cetuximab schedule and in B for the cetuximab-followed-by-gemcitabine schedule. (C) Wilcoxon two-sample test was used to compare schedules (Cet → Gem *vs* Gem → Cet) bioluminescent data obtained by BAR (n = 10 per schedule) and ANlucBCluc reporter (n = 9 per schedule). The increase in bioluminescence activity refers to an increase in caspase-3 activity.

using these interpolated data from tumors that have both volume and Akt measurements (n = 9 per schedule).

Results and Discussion

Molecular Imaging of Akt Predicts Responsiveness of Tumors to Therapy

To investigate whether imaging of Akt and the caspase-3 reporter activity could be exploited to determine therapeutic response earlier than conventional anatomic modalities, we generated mice bearing human UMSCC1 xenografts expressing the BAR and caspase-3 reporter (ANlucBCluc) as shown in Figure 1*A*. Once tumors were palpable, mice (n = 5 per group) were treated with either gemcitabine on day 1 followed by cetuximab on day 2 (Gem → Cet) or cetuximab on day 1 followed gemcitabine day 2 (Cet → Gem). Tumors in animals treated with gemcitabine followed by cetuximab were notably smaller when compared with mice treated with cetuximab followed by gemcitabine. Quantification of tumor volumes showed a clear bifurcation in the two groups from day 7 onward (Figure 1*B*). Significant differences in the tumor volumes between the two schedules (Gem → Cet *vs* Cet → Gem) were detected on days 7 and 10 (P = .004 and P < .0001; Figure 1*C*).

In addition to tumor volumes, AKT (BAR) and caspase-3 (ANlucBCluc) bioluminescence was monitored in both groups before therapy and 7 hours after each treatment followed by daily measurements (Figure 2). Representative images of bioluminescence acquisition are shown in Figure 2. Spline function was applied to the observed volume measurements (on days 1, 4, 6, and 10) to interpolate data every 24 hours from day 4 to 10 using the Akt measurements (Table 1). Wilcoxon two-sample tests suggested significant differences were detected from day 6 to day 10 based on volumes and was detected from day 4 to day 10 based on molecular imaging of Akt, respectively (Table 1A). Akt activity was negatively correlated with tumor volume measurements at all the time points where two schedules had significant differences (Table 1B). Interestingly, there was no significant difference in caspase-3 activity at any of the time points analyzed (Figure 2), suggesting that caspase-3–dependent apoptosis may not play a major role in the difference observed between the two schedules. Cetuximab has been reported to modulate autophagic clearance of the cells, and this may partly explain the lack of difference in caspase-3 activity in the two schedules and correlation between tumor volume and caspase-3 activity (data not shown).

In summary, we detected tumor volume differences between these two schedules only at 144 hours but found significance difference in Akt activity at 96 hours. Thus, our results suggest that molecular imaging of Akt has the potential to detect outcome of treatment earlier than using the volume end point (Table 1A). Because multiple oncogenic pathways mediate their effects through Akt, contributing to the central role of Akt in cell proliferation and survival, Akt reporters will greatly facilitate early prediction of efficacy of other molecular targeted drugs that impinge on activity.

Because molecular imaging techniques are noninvasive, repetitive imaging of the same cohort of animals at different time points not only provide a unique method to predict efficacy of a therapeutic regimen but also monitor drug-target interactions and pharmacodynamics of drug in real time. When compared with the cumbersome conventional methods that are based on euthanizing of animals at different time points for assessing changes in signaling, molecular imaging provides a significant reduction in pain and the cost associated with *in vivo* investigations. A major limitation of such genetic sensors is that the reporter needs to be transfected into tumor cells to monitor its activity. Thus, such reporter will have limited utility in clinic. Thus, until nongenetic molecular imaging assays are developed, luciferase complementation–based reporters may be used for target validation and dose and schedule optimization in preclinical models as well as providing for rapid identification of lead compounds from a library using cell-based, high-throughput screening.

Table 1. Early Prediction of Therapeutic Response by Molecular Imaging of Akt Activity When Compared with Anatomic Changes within the Tumor.

(A) *P* Based on Interpolated Tumor Volume and AKT or Caspase Photon Counts

	T96	T120	T144	T168	T192	T216	T240
Volume	0.34	0.08	0.01	0.01	0.005	0.004	0.005
AKT	0.01	0.01	0.01	0.02	0.01	0.01	0.02
Caspase	NA	NA	NA	NA	NA	NA	NA

(B) Correlations Based on Observed AKT and Tumor Volume Measurements

	T24	T96	T168	T240
T25	0.02	0.09	−0.04	−0.13
T96	0.08	0.19	−0.43	−0.6
T144	−0.2	−0.18	−0.64	−0.55
T240	0.04	0.06	−0.3	−0.44

Spline function was applied to the observed volume measurements (at 24, 96, 168, and 240 hours) to interpolate volumes every 24 hours until 240 hours. Similarly, Akt measurements were interpolated every 24 hours from 96 to 240 hours using the observed Akt measurements (at 25, 96, 144, and 240 hours). (A) Wilcoxon two-sample tests were used to compare schedules (cetuximab → Gem *vs* Gem → cetuximab) using these interpolated data from tumors that have both tumor volume and Akt measurements (n = 10 per schedule). Caspase data were not interpolated because no significant difference was detected for all time points. Observed correlation between actual tumor volumes and relative bioluminescent Akt activity are presented in B. Because an increase in bioluminescent AKT reporter activity is monitored when the Akt kinase is inhibited, the negative value in B represents this inverse relationship between Akt kinase (or BAR) activity.

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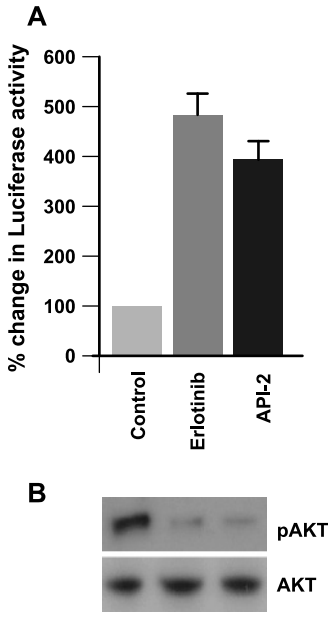

Figure W1. Characterization of Akt reporter in UMSCC1 cells. (A) UMSCC1 cells stably transfected with Akt reporter were treated with either 2 μM erlotinib or 10 μM API-2. Change in the bioluminescence activity was measured and plotted as percent change over pretreatment values. Mean values from three independent experiments are plotted. Error bars, SEM. (B) Inhibition of Akt activity was confirmed by immunoblot analysis of the cell lysate 1 hour after treatment as shown in A using antibodies specific for phospho-Akt or total Akt.

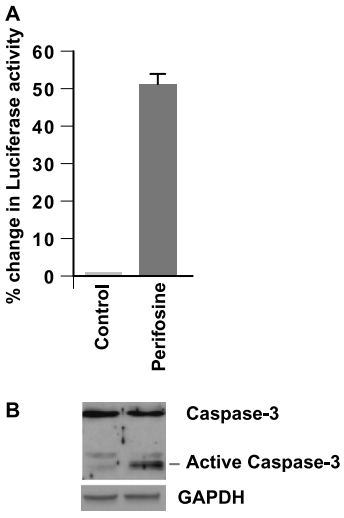

Figure W2. Characterization of caspase-3 reporter in UMSCC1 cells. (A) UMSCC1 cells stably transfected with caspase-3 reporter were treated with perifosine (9 μM) or vehicle, and bioluminescence was measured. Data are derived from three independent experiments and are plotted as described in Figure W1. (B) Activation of caspase-3 was confirmed by immunoblot analysis of the lysate from this experiment using antibodies specific for active caspase-3.