

# AKT expression is associated with degree of pathologic response in adenocarcinoma of the esophagus treated with neoadjuvant therapy

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**Background:** Neoadjuvant chemoradiation (NCRT) has become standard in the treatment of locally advanced esophageal adenocarcinoma (EAC) with survival correlated to degree of pathologic response. The phosphatidylinositol 3 kinase (PI3K)/protein kinase B (*AKT*)/mTOR pathway plays an important role in tumorigenesis and resistance. We sought to elucidate the role of this pathway in patients with EAC who received NCRT.

**Methods:** After IRB approval, a prospective trial was initiated in which patients with EAC underwent endoscopic biopsies of normal and tumor tissue prior to instituting NCRT. Patients then proceeded to esophagectomy. The pre-treatment tissues underwent gene expression profiling. SAM method was used to analyze expression of *AKT* within normal and tumor tissue. Expression was then correlated to degree of pathologic response.

**Results:** One-hundred patients were consented for the study, of which 67 met final eligibility. Nineteen patient's tumors ultimately underwent gene expression profiling via microarray. The differential expression of all *AKT* isoforms in tumor tissue was markedly overexpressed compared to normal tissue ( $P=6 \times 10^{-5}$ ). There were 3 patients designated as pNR, 6 as pPR, and 10 as pCR. Partial and non-responders had higher expressions of *AKT* compared to pCR with the non-responders consistently illustrating the highest expression of *AKT* ( $P=0.02$ ). There was a significant correlation between individual isoforms of *AKT-1*, *AKT-2*, and *AKT-3* and degree of pathologic response ( $P=0.002$ ,  $0.04$ , and  $0.04$  respectively).

**Conclusions:** *AKT* is overexpressed in patients with AC of the esophagus. Moreover, pathologic response to NCRT may be correlated with degree of *AKT* expression. Additional data is needed to clarify this relationship to potentially add targeted therapies to the neoadjuvant regimen.

**Keywords:** *AKT* expression; esophageal cancer; neoadjuvant therapy; esophagectomy; postoperative outcomes

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## Introduction

It is estimated that in the United States there were 17,990 new cases of esophageal cancer with approximately 15,210 deaths of disease in 2013 (1). Worldwide esophageal cancer is the 5<sup>th</sup> leading cause of cancer death in males and the 7<sup>th</sup> in female population combining for 400,000 deaths (2). The 5-year survival for all stages is low and estimated to be 16%;

however, for localized disease in the esophagus the survival may reach 37% (3,4). While surgical resection has remained the mainstay of treatment for esophageal cancer, outcomes of patients treated with surgery alone have been dismal (5-7).

The role of neoadjuvant chemotherapy with or without the addition of radiation for the treatment of localized esophageal cancer has been investigated in an attempt to improve oncologic outcomes (8-17). While there

have been some which demonstrated increased rates of complete pathologic response along with improved overall survival (OS) (18), these results have not been generally reproducible; possibly related to heterogenic patient populations and limited power to demonstrate differences in survival (17,19,20). Neoadjuvant therapy with chemoradiation (NCRT) has the potential to significantly downstage esophageal cancers and thus increase complete resection (R0) rates even in the setting of locally advanced disease (4,21-25). However, despite excellent OS in patients with pCR, the benefit of NCRT in patients whose tumors show pathologic non response remains unclear. Given the potential morbidity, delay in surgical resection, and costs associated with NCRT, it is critical to identify subpopulations of patients who may or may not benefit from such treatment. Conversely identifying those who would have a complete pathologic response may eliminate the necessity for an operation altogether.

The phosphatidylinositol 3 kinase (PI3K)/protein kinase B (*AKT*)/mTOR signaling pathway plays an important role in regulating tumor cellular apoptosis, protein translation, and survival (26). PI3K is activated by receptor tyrosine kinases, and activation of these tyrosine kinases leads to allosteric joining to the cellular membrane and subsequent tyrosine phosphorylation of the regulatory subunit of PI3K. PI3K converts phosphatidylinositol 2 phosphate (PIP2) to phosphatidylinositol 3 phosphate (PIP3) (26,27). *AKT* is activated by phosphorylation at Thr308 by PIP3 and at Ser473 by mammalian target of rapamycin (mTOR) as a part of the mTOR complex (mTORC) (27). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a well-described negative regulator of the PI3K/*AKT* signaling pathway, which functions as a tumor suppressor gene by induction of G1 phase cell cycle arrest through decreasing the levels of cyclin D1 (28). Patients with high expression of activated (phosphorylated) *AKT* are reported to be resistant to radiation therapy (29). The purpose of this study was to examine the differential expression of *AKT* in patients with esophageal cancer and to correlate this expression with response to neoadjuvant chemotherapy and radiation.

## Methods

### *Patient selection and specimen collection*

After IRB approval, a prospective trial was initiated in which patients with locally advanced esophageal adenocarcinoma

(EAC) requiring NCRT were consented for endoscopic biopsies of normal and tumor tissue prior to instituting therapy. All patients were staged with seemingly operable (non-metastatic) esophageal AC via endoscopic ultrasound. All patients had greater than stage II disease (T2N0 or greater according to AJCC standards), which required NCRT. Endoscopic biopsy specimens were obtained from the 19 patients with esophageal AC prior to the initiation of neoadjuvant therapy. In each patient, approximately five biopsy specimens were taken from the tumor and five from the surrounding non-cancerous epithelium. Specimens were frozen immediately at -70 °C for RNA extraction. Extracts of the RNA obtained from each tumor specimen were maintained separately for each patient, while the RNA extracted from the 'normal' esophageal mucosa was pooled for each patient in order to serve as a reference probe (control) for comparison with the cancer.

### *Neoadjuvant therapy and pathologic response*

Patients underwent neoadjuvant chemotherapy with infusional 5-FU and two courses of cisplatin (on day 1 and 28) concomitantly with external beam radiation to a total dose of 50.4 Gy. Patients were treated for a period of 6 weeks, and then underwent re-staging. Patients deemed non-metastatic and medically fit for surgery then underwent esophagectomy. Based upon pathologic review of the resected specimen, patients were categorized into one of three groups: pathologic complete response (pCR) was defined as no residual tumor, partial pathologic response (pPR) as a 50% reduction in tumor size or nodal down-staging, and non-response (pNR) as no difference between pre-operative and post-operative stage based upon endoscopic ultrasound. Board certified pathologists with specialization in gastrointestinal malignancies reviewed all specimens and determined the final pathologic response.

### *Gene expression*

The tissues underwent gene expression profiling using the Affymetrix 133 plus 2.0 Gene chip. Significance analysis of microarrays (SAM) method was used to analyze significant differential expression of *AKT* within normal and tumor tissue. Differential expression of *AKT*, and individual isoforms *AKT-1*, *AKT-2*, and *AKT-3* were investigated. Normal tissue expression was compared to tumor tissue expression of *AKT* and individual isoforms. Correlation of

**Table 1** Patient characteristics

Variables	Data (n, %)
Sex	
Male	17 (89.5)
Female	2 (10.5)
Age	66.3±9.7
Clinical T stage	
T2	1 (5.3)
T3	9 (47.4)
T4	2 (10.5)
Unknown	7 (36.8)
Clinical N stage	
N0	1 (5.3)
N1	11 (57.9)
Unknown	7 (36.8)
Response to NCRT	
Non	3 (15.8)
Partial	6 (31.6)
Complete	10 (52.6)

NCRT, neoadjuvant chemoradiation.

gene expression of *AKT* and pathologic response was also performed. Pathologic complete responders were compared to those with less than a complete response (pPR and pNR).

### Statistical analysis

Tumor and pooled normal tissue samples were matched for each patient. A mean difference of expression was calculated. The SAM (15) was used to analyze gene expression data. SAM has been widely used for microarray data analysis with adjustment for multiple simultaneously testing. SAM can be used for a variety of statistical tests, such as paired *t*-test, two-sample *t*-test, ANOVA, and survival analysis. In this study, we used the two-sample *t*-test setting in identifying differentially expressed genes.

## Results

### Demographics

One hundred patients were consented for participation in this study, of which 67 met final eligibility criteria. Nineteen patients with adenocarcinoma had tissue specimens that were analyzed via microarray (5 tumors and 5 normal tissues) for each patient. Patient characteristics are reported in *Table 1*. Seventeen males (89.5%) and

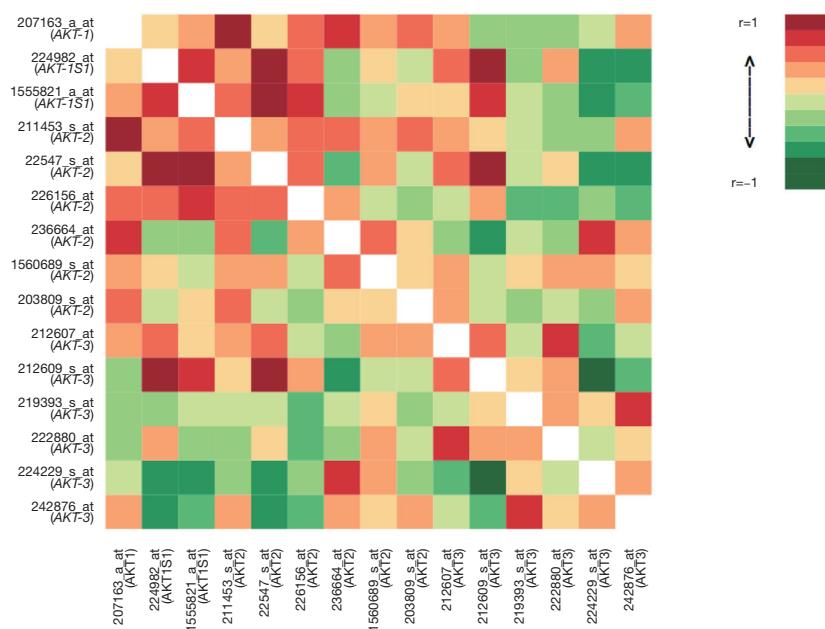
2 (10.5%) females comprised the patient population with a mean age of 66.3±9.7 years. There were 3 (15.8%) non-responders, 6 (31.6%) partial responders, and 10 (52.6%) complete responders. T-stage was known in twelve (63.2%) patients and were T2 or higher. N-stage was also known in 12 patients (63.2%). In the patients with known nodal stage, 11 (92%) were N1, and 1 (7%) was N0. Seven (36.8%) did not undergo endoscopic ultrasound but all had what was deemed locally advanced tumors due to size or clinical symptoms. Five of these patients had a complete pathologic response, and 2 had significant response to therapy but had residual disease and were deemed as partial responders.

### *AKT* expression in tumor vs. normal tissue

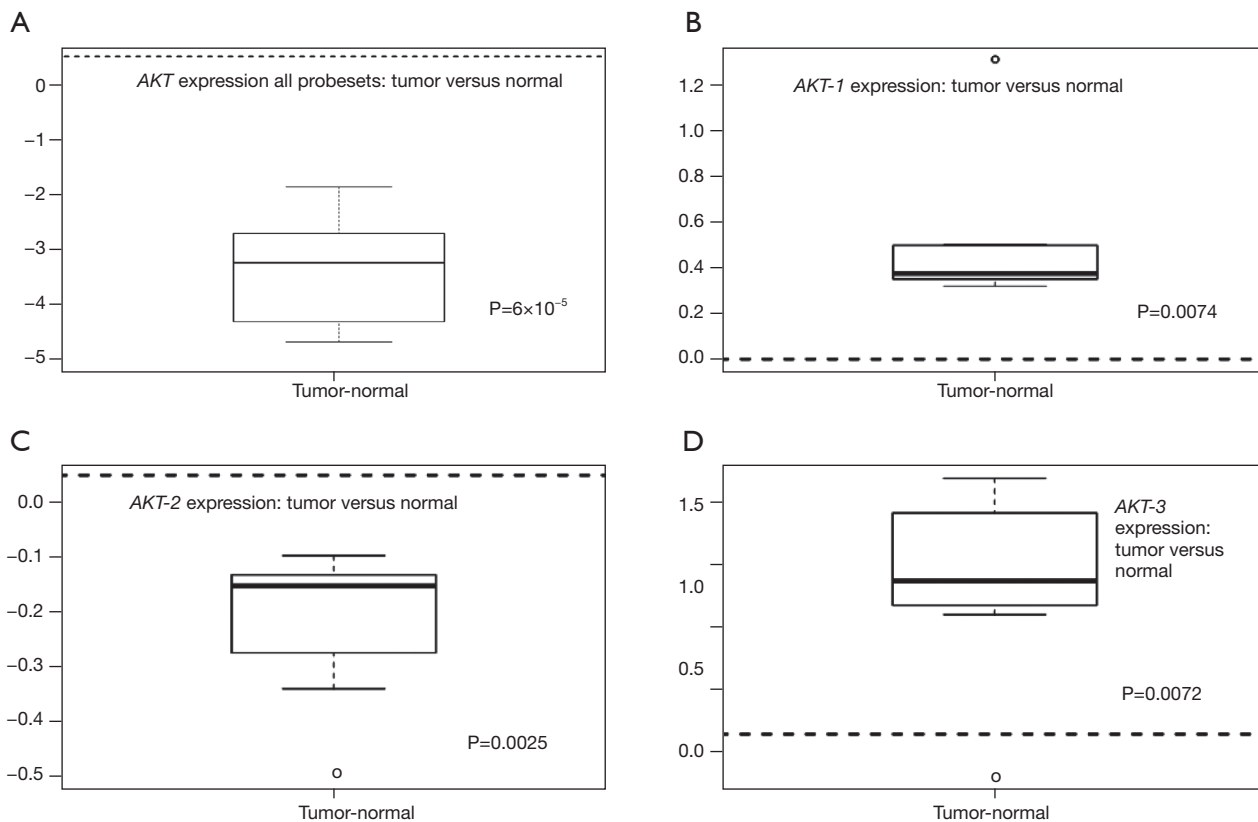
Differential gene expression analysis was used to compare overall *AKT* and individual isoform expression of *AKT-1*, *AKT-2*, and *AKT-3* in tumor and normal tissues. *Figure 1* illustrates the heat map of all *AKT* probesets. Significant clustering of gene expression for *AKT* in tumor tissues was demonstrated ( $P=3\times 10^{-4}$ ). Differential expression of all isoforms for *AKT* revealed marked overexpression in tumor tissues compared to their normal tissue counterparts (*Figure 2A*,  $P=6\times 10^{-5}$ ). As illustrated in *Figure 2B-D*, individual analysis of *AKT-1*, *AKT-2*, and *AKT-3* also revealed overexpression in tumor tissue compared to their normal tissue cohorts. There was significant overexpression of *AKT-1*, with a mean difference of 0.53 between tumor and normal tissues ( $P=0.0074$ ). The mean differences in expression of *AKT-2* between tumor and normal tissues were 0.27 ( $P=0.0025$ ). Differential expression analysis also found significant differences in *AKT-3* expression between the two tissue types of 1.07 ( $P=0.0072$ ).

### Pathologic response and *AKT* expression

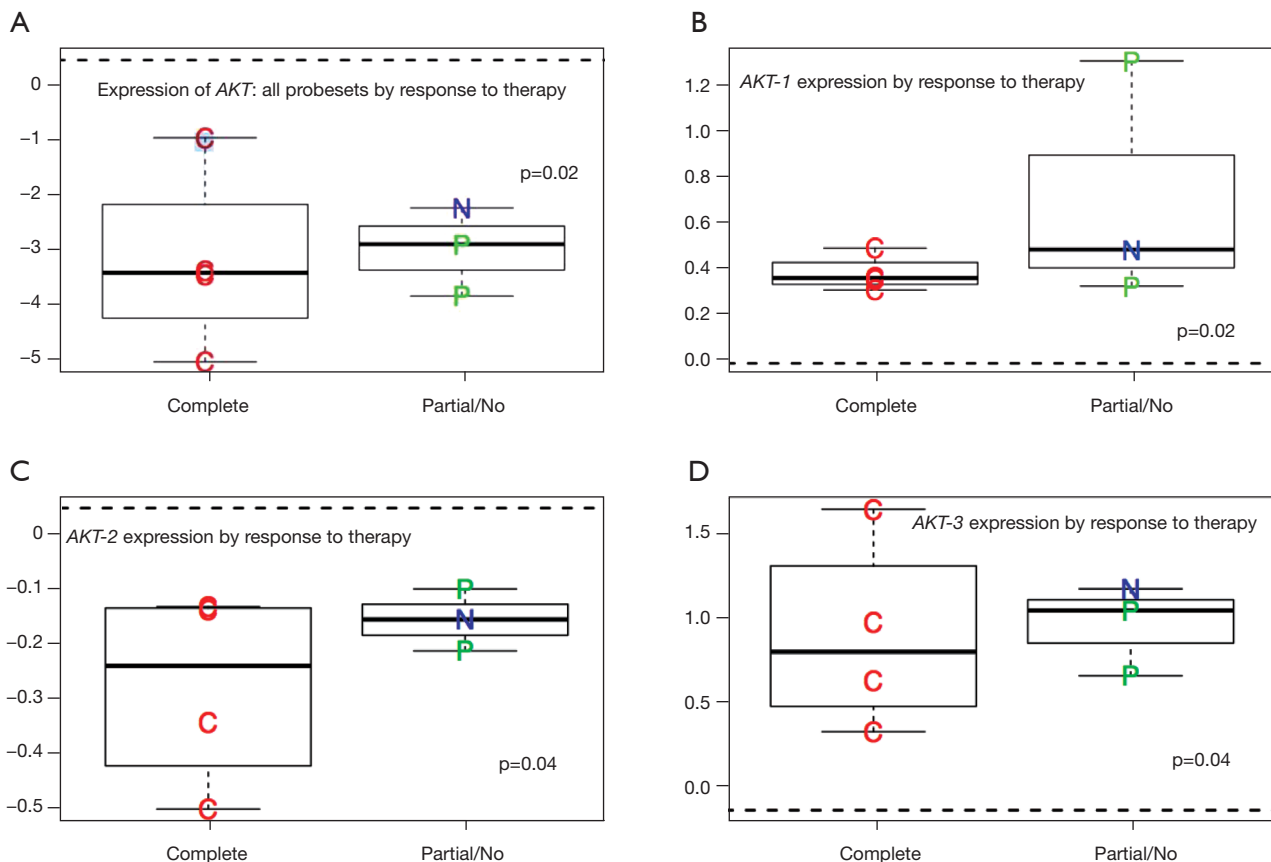
We identified a significant correlation between *AKT* expression and degree of pathologic response. Partial and non-responders had higher expressions of *AKT* compared to complete responders, with the non-responders consistently illustrating the highest expression of *AKT* *Figure 3A*. The mean difference of expression was 3.35 ( $P=0.02$ ) for pCR compared to pPR/pNR. Over expression of *AKT-1*, *AKT-2*, and *AKT-3* was also shown to be a predictor of pathologic response to therapy (*Figure 3B-D*). *AKT-1* demonstrated a mean difference of expression of 0.39,  $P=0.002$ ; *AKT-2* demonstrated a mean difference of expression of 0.32,  $P=0.04$ , and *AKT-3* demonstrated a mean difference of



**Figure 1** Heat map of all *AKT* probesets ( $P=3 \times 10^{-4}$ ).



**Figure 2** (A) *AKT* expression in normal *vs.* tumor tissues for all isoforms. *AKT* is overexpressed in tumor tissues (mean diff. = -3.84;  $P=6 \times 10^{-5}$ ); (B) *AKT-1* expression (mean diff. = 0.53;  $P=0.0074$ ); (C) *AKT-2* expression (mean diff. = -0.27;  $P=0.0025$ ); (D) *AKT-3* expression (mean diff. = 1.07;  $P=0.0072$ ).



**Figure 3** (A) *AKT* all isoforms expression correlated to pathologic response to NCRT (mean diff. =-3.35 for pCR vs. pPR/pNR  $P=0.02$ ); (B) correlation between *AKT-1* expression and pathologic response to NCRT (mean diff. =0.39;  $P=0.002$ ); (C) correlation between *AKT-2* expression and pathologic response to NCRT (mean diff. =-0.32,  $P=0.04$ ); (D) correlation between *AKT-3* expression and pathologic response to NCRT (mean diff. =0.58,  $P=0.04$ ). NCRT, neoadjuvant chemoradiation; pCR, pathologic complete response; pPR, partial pathologic response; pNR, pathologic non-response.

expression of 1.01,  $P=0.04$ .

Interestingly, the expression of *AKT* in the tumor tissues exhibiting a pCR more closely mimicked expression of *AKT* in normal tissue. *AKT-1* demonstrated a minimal mean difference of expression of 0.3 ( $P=0.1$ ) and *AKT-2* 0.15 ( $P=0.09$ ). Whereas differential expression of *AKT-3* in pCR tumor tissues remained significantly overexpressed with a mean difference of expression of 0.7 ( $P=0.002$ ) compared to normal tissues.

## Discussion

We have illustrated that patients with EAC have significant overexpression of *AKT* in their tumor tissues compared to the normal tissue expression of *AKT*. Additionally,

individual *AKT* isoforms exhibited overexpression of *AKT-1* ( $P=0.0074$ ), *AKT-2* ( $P=0.0025$ ), and *AKT-3* ( $P=0.0072$ ) in the tumor tissues. The correlation of the degree of expression between pathologic responses to NCRT demonstrated a linear relationship between the higher expression of *AKT* and decreasing degree of pathologic response. Partial and non-responders had higher expressions of *AKT* compared to pCR with the non-responders consistently illustrating the highest expression of *AKT*. Individual probesets were also found to correlate with degree of pathologic response. We demonstrated a significant overexpression of *AKT-1*, *AKT-2*, and *AKT-3* in partial/non-responders versus complete responders.

The overexpression of *AKT* in tumor tissues and the positive correlation between *AKT* expression and decreasing

pathological response to chemoradiation indicate the importance of the PI3K/AKT/mTOR pathway in the progression of esophageal AC and its impact on the prognosis of patients. We previously reported our series of 347 patients who underwent esophagectomy with curative intent in an attempt to assess the impact of pathologic response to neoadjuvant chemoradiation (NCRT) on OS (30). Patients with a pCR to NCRT were found to have a significantly higher 5-year disease-free survival (DFS) and OS compared to patients with partial response (pPR) and no response (pNR) (52% and 52%, respectively, compared to 36% and 38% in pPR and 22% and 19% in pNR,  $P < 0.0001$ ). This was consistent with other studies that have shown dramatic survival benefits in patients with complete or pPRs to NCRT (31-33).

The PI3K/AKT/mTOR pathway regulates a number of cell processes involved in tumor progression, including proliferation, invasion, and apoptosis (34). This signaling pathway is activated in malignancies and plays an important role in the development of resistance to chemotherapeutic agents (35). A significant correlation between treatment outcome in various malignancies and AKT expression has been identified by Bussink *et al.* (34). Rapamycin was initially considered as a promising modality for blocking mTOR phosphorylation in several cancer types; however, cancer patients with high expression AKT may have little response to mTORC1 inhibitors (36).

The PI3K/AKT/mTOR pathway also plays a significant role in the progression of a number of other malignancies (37). Chung *et al.* observed activated AKT expression in 84.2% of extrahepatic cholangiocarcinoma cases. They found significant increases in p-AKT and p-mTOR expressions from normal biliary epithelial cells to infiltrating malignant neoplastic epithelial cells. Moreover there was a clear correlation between survival of patients with extra-hepatic cholangiocarcinoma and specific relative expression level of active AKT and mTOR (37).

Over-expression of AKT has also been found to be associated with the development of resistance to radiation therapy, through mechanisms including intrinsic radioresistance, tumor-cell proliferation, and hypoxia (29,34). A review on the activation of the PI3-K/AKT pathway and the implications for radioresistance mechanisms by Bussink *et al.* has demonstrated that pAKT expression is an independent prognostic factor for survival in patients with head and neck cancers (34). David *et al.* in their series of 61 patients with non-small cell lung cancer found a statistically significant decrease in survival between

patients with pAKT over-expression and patients with weak expression (38). Le Page *et al.* investigated the correlation between the expression and localization of AKT-1, AKT-2, AKT-3, phospho-AKT proteins and the clinicopathological parameters in 63 prostate cancer specimens. They found that more than 60% of tumor tissues overexpressed AKT-1, AKT-2 or AKT-3 and that AKT-1 expression was associated with a higher risk of PSA recurrence and shorter PSA recurrence interval.

Resistance to radiation therapy is a potential explanation reason for the low survival rates and high incidence of local recurrence for esophageal cancer (39). The 5-year survival for patients with esophageal cancer is estimated at 10-20% (39). Therefore is important to identify strategies for reducing chemo and radioresistance and thereby improve the effectiveness of neoadjuvant therapy. Inhibition of the PI3K/AKT/mTOR can augment the effectiveness of radiation by blocking cellular defense mechanisms induced by radiation (34). Hildebrandt *et al.* found that expression of AKT1 and AKT2 were associated with an increased risk of recurrence in patients with esophageal cancer (adenocarcinoma or squamous). In addition, AKT2 was associated with a poor response to chemoradiotherapy treatment (35). In our data, we corroborated this finding that AKT-2 was associated with poor response to NCRT. However, we also demonstrated that other isoforms were also predictive of response. Since AKT plays a significant role in anti-apoptotic pathways, agents that block AKT activation could play an important role in the development of chemotherapeutic treatments that improve outcomes for patients with esophageal AC.

Le Page and colleagues evaluated the radiosensitizing effect of a COX-2 inhibitor, NS398, and its mechanism in radioresistant esophageal cancer cells. They found that NS398 blocks AKT activation and induces apoptosis in Eca109R50Gy cells and therefore increased radiosensitivity in these cell types (39,40). While the present study did not correlate survival and AKT expression, patient's exhibiting less than a complete response to NCRT had the highest expressions of AKT. Given the poor prognosis of patient's with a non response, it is reasonable to assume that AKT over expression may impact survival in these patients indirectly by inhibiting their potential for maximal response to therapy.

## Conclusions

AKT over expression has been shown to correlate to

outcomes in various malignances. In our analysis of patients with EAC, we found that *AKT* is overexpressed in tumor tissues compared to normal tissue *AKT* expression. Additionally, all isoforms of *AKT* exhibited over expression. Moreover, pathologic response to NCRT correlated with degree of *AKT* expression. Our results support the need for developing targeted agents that will decrease chemotherapy and radiation resistance and thereby increase efficacy by regulating *AKT* expression through the PI3K/*AKT*/mTOR pathway.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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