The Akt Pathway as a Biomarker in Carcinogenesis
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Abstract

Problem Addressed: The only definitive and point for studies of chemoprevention is the determination of the incidence of primary and secondary cancers. Such an endeavor would be a multi-year study with thousands of subjects and would have tremendous costs. The identification and validation of intermediate endpoints would be an important step in evaluating chemopreventive agents. We have previously noted that carcinogenesis, an exciting opportunity with promising chemopreventive effects in HNSCC, inhibited the Akt pathway. Hence we wanted to determine if there was a difference in expression of pAkt in tumors and adjacent mucosa of HNSSC patients when compared to non cancer patients.

Methods and Measures: 24 HNSSCC and 23 non-cancer (sleep apnea) patient samples were analyzed by western blot for expression of phosphorylated Akt (pAkt)(Ser473). The SCC samples include tumors and adjacent mucosa. Results: No expression of pAkt was seen in mucosa of 12/16 non-cancer patients while tumors and adjacent mucosa express pAkt in 15/16 HNSSCC patients (p<0.05).

Conclusions: Biomarkers in the Akt/mTOR pathway may be sensitive as Akt/mTOR is activated in 99% of HNSSCC and non mucosa suggesting Akt is a good biomarker for a chemopreventive agent.

Clinical Significance of Study: This preferential activation of the Akt pathway in HNSSCC compared with the non-cancer patients could be useful in the design of clinical trials with Akt/mTOR inhibitors.

Introduction

Signaling pathways upstream and downstream of mTOR appear dysregulated in human cancers [1-3], suggesting that this pathway plays an essential role in cancer in the transformed phenotype. Alterations in the PI3K-mTOR pathway in human cancers include loss of PTEN phosphate function, amplification of AKT, mutations of tubulins and serine kinases (TSC1/2), and overexpression of eIF4E or S6K. Overexpression of eIF4E is functionally active in tumors and some tumor free margins through activation of the Akt/mTOR signaling pathway [4]. In earlier studies on surgical margins, as the degree of dysplasia increased there was an increase in overexpression of eIF4E in the surgical margins of HNSSCC patients [5]. However, while there was a significant association between dysplasia and overexpression of eIF4E, overexpression of eIF4E was an independent predictor of recurrence and not the degree of dysplasia. Hence, activation of the Akt/mTOR pathway in premalignant lesions of the oral cavity could provide an important intermediate endpoint in trials with novel chemopreventive agents.

Tissue Samples and Controls: Tumor and adjacent mucosa were obtained from 34 cancer patients and normal mucosa from 23 patients who have had their soft palate excised for sleep apnea under Institutional Review Board approved protocol. Tissues were obtained in the operating room immediately after resection of the primary tumor or ura and were immediately frozen in liquid nitrogen and stored at -80°C.

Western Blot Analysis: Soluble proteins were extracted by suspending tissues (~5mg) in 200 l of lysate buffer, placed on ice for 30 minutes, centrifuged at 14,000 rpm at 4°C for 30 minutes, and the supernatant stored at -80ºC. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Lysates were separated on 7% or 12% SDS PAGE gels, proteins transferred to a nitrocellulose membrane (Amersham, Biosciences, Germany) and the membranes blocked with 5% non-fat milk in TBS (0.05%, Tween 20, 0.1% Tris-HCl pH 8.0, 150 mM NaCl) for 1 hour. Membranes were subjected to western blotting with the following primary antibodies; rabbit polyclonal anti-4E binding protein 1 (4EBP1) (1:3500 dilution), rabbit polyclonal anti-phospho-Akt (Ser473; 1:250 dilution), phospho-mTOR (2448; 1:500 dilution), and rabbit anti-actin (1:1000 dilution). Membranes were subjected to western blotting with the following primary antibodies: rabbit polyclonal anti-Akt pTyr1068, rabbit polyclonal anti-4E binding protein 1 (4EBP1) (1:3500 dilution), rabbit polyclonal anti-phospho-Akt (Ser473; 1:250 dilution), phospho-mTOR (2448; 1:500 dilution), and rabbit anti-actin (1:1000 dilution). The membranes were incubated overnight with the primary antibodies at 4°C. The membranes were then rinsed three times with TBS and incubated with goat anti-rabbit IgG alkaline phosphatase conjugates (1:2500 dilution) and rabbit anti-Akt (1:1000 dilution) antibody were then incubated with anti-Akt antibody. Akt antibodies were then visualized using the ECL system (Amersham, Arlington Heights, IL). The membranes were then stained with Coomassie Blue to verify equal loading.

Statistical Analysis: A Mann-Whitney U test was performed to examine the difference in the levels of each biomarker in cancer and non-cancer patients.

We demonstrate here that the Akt/mTOR pathway can serve as potential biomarkers in HNSSCC, consistent with a recently published micrometastasis study [6]. We explored whether differences existed in expression between cancer and non-cancer patients by western blot in the Akt/mTOR pathway as all previous studies were done by immunohistochemistry [7,8] and the need to validate this method for precancer biopsies where the tissue sample is often small is important as the background effect of staining with HIC is significant in smaller specimens. Lippman [9] proposed criteria for biomarkers in tobacco related epithelial carcinogenesis and our previous data demonstrates that Akt/mTOR meet these requirements [7,8]. In Figure 2 we present an analysis of malignant (lanes 5, 8, 11, and 12) and non-cancer (patients with sleep apnea) (lanes 1-4 and 7-10) patient samples. The malignant samples include tumors (T) and adjacent mucosa (A). Very little expression of p-Akt was seen in mucosa of non-cancer patients (0.88 ± 1.54) while tumors (2.29 ± 3.30) and some adjacent mucosa (2.65 ± 3.02) express high levels of pAkt (Fig 2 and 3). Because we wanted to determine which of the targets in the Akt/mTOR pathway showed consistent differences in expression between normal and cancerous tissue by western, we also explored pS6, p-mTOR, and p-4EBP1 in these same tissues. Tumor (0.27 ± 0.44) and adjacent mucosa (0.50 ± 0.42) had lower levels of p-4EBP1 than non-cancer patients (0.84 ± 0.36), while no statistically significant difference was observed with pS6 (0.30 ± 0.20), adjacent mucosa (0.74 ± 0.61), and non-cancer patients (2.82 ± 2.36), or p-mTOR/tumor (0.86 ± 0.69), adjacent mucosa (1.36 ± 1.03), and non-cancer patients (0.87 ± 0.86).

Results


Figure 2. Representative western blots of tissue samples from sleep apnea non-cancer patients (lanes 1-4 and 7-10), and cancer patients (5, 8, 11, and 12) tumor (T) and adjacent (A) mucosa probed with indicated antibodies.

Figure 3. Statistical analysis of western. Tumor samples had significantly higher p-Akt (p<0.04) than non-cancer samples. Adjacent mucosa had significantly higher p-Akt than tumor mucosa (p<0.04). Mean ± SEM.

Conclusions

Identifying biomarkers as intermediate endpoints has become an important step when evaluating chemopreventive interventions. Our previous studies by immunohistochemistry showed that these markers are expressed only in the basal cell layer of malignant oral mucosa, and are absent in the basal cell layer of non-malignant oral mucosa [7,8]. Future studies of pre-malignant oral mucosa are suited for small samples obtained from pre-malignant oral mucosa studies.

Bibliography