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Evaluation of Phospho-AKt Immunohistochemical Expression in Patients with Laryngeal Squamous Cell Carcinoma

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Abstract

Background Akt, is a serine/threonine protein kinase which act as an important regulator of cell proliferation and survival. The Akt complex is upregulated by phosphorylation producing phospho-Akt, which trigger a continued cell proliferation and survival and inhibit apoptosis, thereby promote cell survival.

Objective To evaluate the immunohistochemical expression of phosphorylated Akt (Phospho-Akt) in laryngeal squamous cell carcinoma (SCC) and to be correlated with different clinicopathological parameters.

Methods Phospho-Akt expression was investigated Immunohistochemically in 49 formalin-fixed paraffin embedded laryngeal SCC tissue sections collected from Teaching laboratories - Baghdad Medical City.

Results Phospho-Akt positive immunostaining appears in 57% of samples. Akt activation present in advanced stages of tumors with p value 0.02.

Conclusion The current findings may provide evidence that aberrant expression of Akt contributes to the pathogenesis (mechanism of disease development) of laryngeal SCC.

Keywords Akt, phospho-Akt, immunohistochemistry, laryngeal SCC, larynx

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List of abbreviations: DAB = diaminobenzidine, MSCC = Moderately differentiated squamous cell carcinoma, PBS = Phosphate buffered saline, PSSC = Poorly differentiated squamous cell carcinoma, PTEN = Phosphatase and tensin homolog, SCC = Squamous cell carcinoma, WSCC = Well differentiated squamous cell carcinoma

Introduction

Globally, Cancer of larynx is the second most common respiratory cancer after lung cancer [(1)]. In Iraq, laryngeal cancer constitutes 24.8% of head and neck cancer and 2.47% of all cancers [(2)].

Mortality rate of laryngeal cancer is about two times higher in developing than developed countries [(2)]. This increase is likely attributed to increased exposure to risk factors like smoking, drinking, population aging and increased exposure to industrial carcinogens. In Iraq, laryngeal cancer peak at age 80 years with 21.7 deaths per 100,000 men in 2010. It causes death at the lowest scale at age 30-34 years [(3)]. The highest rate of death for women was less than that of men, which were 2.8/100,000 women [(3)].

Akt or Protein Kinase B (PKB) is a serine/threonine protein kinase that functions as an important regulator of cell proliferation and survival [(4)]. It is comprised in cellular survival pathways, by suppressing apoptotic processes [(5,6)]. The PI3K/Akt kinase pathway is a

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central regulator of cell metabolism, proliferation, and survival and is dysregulated by oncogenic events in a substantial fraction of tumors. Constitutive activation of growth factor receptors, mutation of PI3K, and inactivation of the phosphatase and tensin homolog (PTEN) cause the activation of PI3K signaling in many tumors [7]. Since PI3K has a multitude of downstream targets, including Akt, mutations of which are oncogenic and occasionally present in human tumors. In seriously proliferating tumor cell, the Akt complex is turned on by phosphorylation, which will trigger a continued cell proliferation and cell survival and inhibit apoptosis [6]. Since Akt phosphorylation can hinder apoptosis, and by that promote cell survival, it has been involved as a major factor in many types of cancer [8-11].

These changes lead to constitutionally active survival signaling and that increase the insensitivity of tumor cells to apoptosis induction [6]. Therefore, the present study is aimed to figure out the role of Akt activation in laryngeal squamous cell carcinoma (SCC) pathogenesis.

Methods

Tissue samples

Forty-nine tumor specimens of laryngeal SCC were obtained from archived formalin fixed paraffin-embedded tissue samples of surgically resected tumors in National Center for Educational Laboratories in City of Medicine (Baghdad-Iraq). Ethical agreements were obtained from Baghdad directorate of health. Tumors were classified into three grades: well differentiated squamous cell carcinoma (WSCC), moderately differentiated squamous cell carcinoma (MSCC), and poorly differentiated squamous cell carcinoma (PSCC). Clinicopathological parameters of the larynx cancer patients were shown as age, gender, tumor grade and stage.

Immunohistochemical analysis

Immunohistochemical staining for Phospho-Akt in paraffin embedded tissue sections was performed using indirect biotin-avidin system. Slides deparaffinization processed in three changes of xylene for five minutes each. Slides were transferred to ascending grades of ethyl alcohol, followed by washing with distilled water. To unmask the antigenic epitope, antigen retrieval was performed by placing the slides in glass coplin jar filled with sodium citrate buffer (pH 6.0) in a microwave oven at 90 °C for 20 minutes. Slides were allowed to cool at room temperature. 0.3% of hydrogen peroxide were added then incubated for 30 minutes to block the activity of endogenous peroxidase. Slides were next Incubated with rabbit polyclonal phospho-Akt (Ser 473) antibody (Thermo Scientific, USA) in a humidified chamber over night at 4 °C. The slides were rinsed three times with PBS then incubated with a biotinylated secondary antibody for 30 minutes. The reaction product was developed using diaminobenzidine (DAB) as chromogen and observed under the microscope for development of brown color. The color reaction was stopped by dipping the slides in distilled water. Sections were then counterstained with Harris-haematoxylin. Slides were dehydrated through three changes of 99% alcohol for 5 minutes each and mounted using DPX slide mounting medium. Adjacent normal appearing epithelium within the tissue sections served as a positive internal control. Representative areas of each tissue sections were selected and were counted in 5 fields at x400 magnification in each section. Sections were considered immunopositive when more than 10% of the tumor cells had clear evidence of immunostaining [12]. For negative controls, similar procedure was followed without primary antibody.

Statistical analysis

The chi-square test was used to determine the correlation of Akt phosphorylation with different clinicopathologic parameters [13].

Results

The clinical and pathological parameters of 49 cases of laryngeal carcinoma are shown in table 1. Out of these, 14 (28.5%) were well

differentiated SCC, 20 (41%) were moderately differentiated SCC and 15 (30.5%) were poorly differentiated SCC. Stages of tumor were distributed as following: 31 (64%) with stages I&II, and 18 (36%) with stages III&IV. Out of 49 cases, 39 (80%) cases were fifty years old and above and 10 cases (20%) were less than fifty years old.

Phospho-Akt expression

The relationship between phosph-Akt expression and clinicopathological characteristics of tumor samples is shown in table (1). Phospho-Akt immunostaining was positive in 28 (57%) out of 49 samples. Among fifty years old and above patients, 23 (59%) cases were phospho-Akt positive out of 39 cases. In relation with patients' gender, out of 45 males, 26 (92%) show phospho-Akt positive staining and 19 males (90%) were negative (Table 1). However, no significant correlation was observed between both age, gender and phospho-Akt overexpression. Immunostaining for phospho-Akt was both nuclear and cytoplasmic staining (Figure 1A&B). Over-expression of phospho-Akt was 9 (32%), 11 (39%) and 8 (29%) out of 14 cases well differentiate SCC, 20 cases moderately differentiated SCC and 15 cases poorly differentiated SCC respectively. However, the observed differences failed to achieve the level of statistical significance. Our data showed that phosph-Akt overexpression was observed in 14 (50%) cases in advanced stages of tumor (stage III &IV). In earlier stages (stage I&II) of laryngeal carcinoma, phospho-Akt was not rare since it occurred in 14 (50%) of cases. Our marker showed a statistically significant correlation with tumor stage with p value 0.02 (table 1).

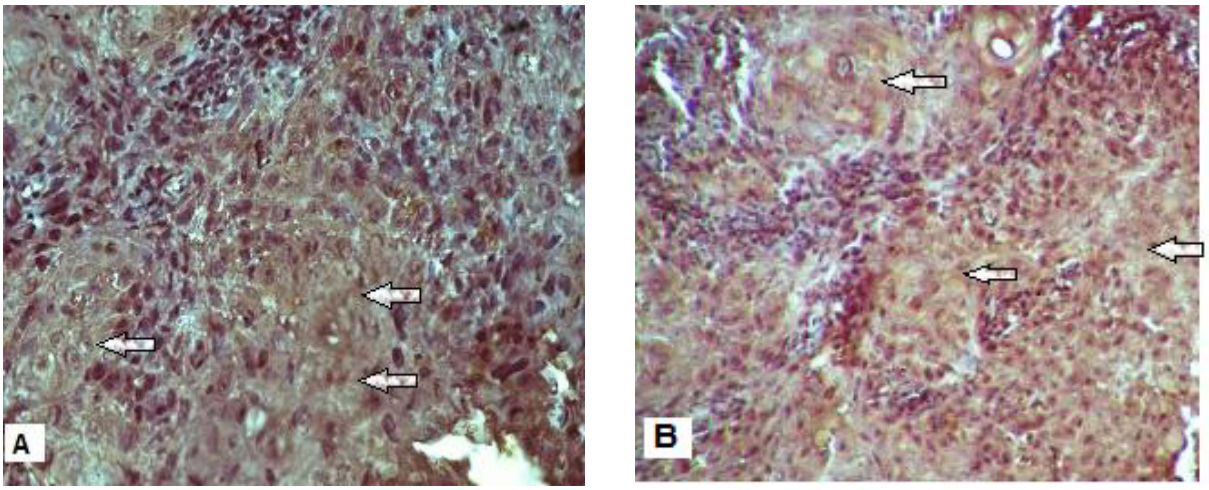

Figure 1. Representative photographs of Immunohistochemical staining for phospho-Akt expression. A&B: laryngeal Tumor showing strong positive immunostaining (both cytoplasmic and nuclear) for phospho-Akt at Ser 473 in well differentiated and poorly differentiated squamous cell carcinoma respectively (400x). Counter stain: hematoxylin

Discussion

Many approaches had been applied to avoid laryngectomy for patients with high stages of laryngeal SCC using chemotherapy and radiotherapy. However, these treatments are highly toxic, therefore, tumor understanding on molecular basis is essential in the treatment of laryngeal carcinoma. The present study was to determine the possible relevance of phospho-Akt overexpression in clinicopathological features of laryngeal SCC and its role in tumor development in order to provide basis for cancer understanding and treatment.

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Table 1. Phospho-Akt expression and its association with clinicopathological parameters in SCC of the larynx

Clinicopathological parameters		Total n=49	Phosho-Akt positive n =28 (57%)	Phospho-Akt negative n =21 (43%)	P value
Age (yr)	≥ 50 < 50	39 (80.0%) 10 (20.0%)	23 (82.0%) 5 (18.0%)	16 (76.0%) 5 (24.0%)	0.61 (NS)
Gender	Male Female	45 (92.0%) 4 (8.0%)	26 (92.0%) 2 (8.0%)	19 (90.0%) 2 (10.0%)	0.76 (NS)
Tumor grade	W M P	14 (28.5%) 20 (41.0%) 15 (30.5%)	9 (32.0%) 11 (39.0%) 8 (29.0%)	5 (24.0%) 9 (43.0%) 7 (33.0%)	0.81 (NS)
Stage	I & II III&IV	31 (64.0%) 18 (36.0%)	14 (50.0%) 14 (50.0%)	17 (81.0%) 4 (19.0%)	0.02 (S)

Data are number (percentage) of patients; W well differentiated, M moderately differentiated, P poorly differentiated; NS: non significant; S: significant (p value ≤ 0.05)

In this study, it was found that phospho-Akt overexpression was not uncommon event since it occurred in 57% of examined cases. A significant correlation was remarked with the tumor stage (p value 0.02) as shown in table (1).

The act of Akt activation in cancer progression has been assessed in many kinds of tumors like ovarian cancer [8], head and neck cancer [9], tongue cancer [10], and prostate cancer [11]. Many studies of Akt expression in prostate cancer tissues revealed that tumor progression in western populations was significantly correlated with Akt upregulation [15,16]. Le Page et al. explore the localization and expression of the Akt three isomers, proposing a distinct act of Akt-1 expression as a prognostic marker in prostate cancer [17]. In laryngeal cancer, fewer studies were done on Akt marker. Yu et al. studied the possible prognostic significance of Akt in oropharyngeal SCC patients from the United States and found that Akt activation was associated with adverse patients' outcome indicating that Akt is a promising molecular target [18]. Another study from Netherland used antibodies against phospho-Akt revealed low levels of Akt phosphorylation in laryngeal cancer and it was significantly correlated with lymph node metastases [19], a result that doesn't match our present study. Assessment on the role of Akt overexpression was based particularly on data from developed countries. A study from Iraq on Akt expression in oral cancer revealed positive expression of Akt in 38 out of 40 cases and a statistical significant correlation was found with tumor stage [20]. The present study showed Phospho-Akt expression was both cytoplasmic and nuclear (figure 1). Although no significant statistical correlation was found between age of patients and Akt phosphorylation, phospho-Akt positive immune staining was found in 23 (82%) out of 39 patients who were fifty years old and above. For our knowledge this is the first study from Iraq to evaluate the role of phospho-Akt in laryngeal cancer. According to our scoring parameters, upregulation of Akt was noted in 14 (78%) out of 18 cases with advanced tumor stages (stage III&IV). Suggesting that tumor with phospho-Akt overexpression may be more threatening and aberrant expression of Akt may contribute to the pathogenesis of laryngeal SCC.

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Authors contribution

Dr. Wanas: processed the laboratory research work and interpretation of results. Dr. Mehdi: sample collection and article preparation; Alzubaidi: collected data from available registered records.

Conflict of interest

The authors declare no conflict of interest.

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