

ROLE OF PROTEIN KINASES AND KIRSTEN RAS PATHWAYS IN ORAL SQUAMOUS CELL CARCINOMA AND POORLY DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA IN PUNJAB (PAKISTAN) POPULATION

¹FARHAT KAZMI, MPhil Oral Pathology

²MUHAMMAD MUMTAZ, MDS (Oral & Maxillofacial Surgery)

³M H QAZI, PhD (Louisiana State, USA)

ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) develops by accumulation of multiple genetic alterations, influenced by the patient's genetic predisposition as well as by environmental influences, that includes pan, chaalyia, tobacco, alcohol, chronic inflammation, and viral infection.

This study was carried out to understand the molecular alterations which contribute to the development of OSCC in Pakistani population.

The study was conducted on a sample of 53 patients collected from different hospitals of Lahore.

Results of this study indicate that Akt levels shows higher expression with increase in grades. The value of Akt1 in well differentiated grade 1 tissue is of the order of 0.09+0.00, Akt2 is 0.04+0.00 and Akt3 is 0.02+0.00 while in poorly differentiated tissues the values of Akt 1, Akt and Akt3 are 0.22+0.09, 0.13+0.03 and 0.06+0.01 respectively. kRas is an oncogene which is highly elevated in both grades (well differentiated: 0.09+0.02 and poorly differentiated: 0.13+0.02) in all samples of OSCC.

In conclusion, our data demonstrated that Akt isoforms and kRas significantly control the cancer transition pathway. It is seen that Akt 1 expression rises from 2.5- fold in well differentiated tissues to 3.5-fold in poorly differentiated tissues. The Akt-2 on the other hand shows only 0.5-fold increases from normal tissue in grade 1 tissues, but rises to 4-fold in grade-3 tissue. On the other hand there was no change in Akt-3 as compared to normal in grade I tissues yet, 3-fold increase has been recorded in grade III tissue. The oncogene K-RaS shows consistent increase of the order of 2.5-fold in grade I and 3-fold in grade III. This information combined with histopathological reports can further improve our understanding of the prognosis of oral squamous cell carcinoma.

Key Words: Oral Squamous Cell Carcinoma(OSCC), Protein Kinase Kirsten Ras, Pathways.

INTRODUCTION

Oral cancer (OC) is a subtype of head and neck cancer (HNC) that arises in the oral cavity, from squamous epithelium. It is sixth most common cancer in the world with continuous rise in Asia.^{1,2} Overall, head and neck cancer accounts for more than 550,000 cases annually worldwide.³ In Pakistan, oral squamous cell

¹ Associate Professor & Head of Oral Pathology, University College of Dentistry, The University of Lahore. Res: 121-C, Tariq Block, New Garden Town, Lahore

² Asstt Professor (Oral & Maxillofacial Surgery)

³ Professor & Director, Centre of Research in Molecular Medicine, Vice Chancellor, The University of Lahore

Received for Publication: February 1, 2014

Revision Received: February 8, 2014

Revision Accepted: February 11, 2014

carcinoma is the second most common cancer in males⁴ with the highest prevalence rate in Karachi, followed by Jamshoro, Multan and Peshawar.⁵

Oral squamous cell carcinoma arises from oral epithelium and is usually an extremely aggressive tumor whose biological behavior is quite unpredictable having poor prognosis and high recurrence rate.⁶ In this cancer, genetically some cells acquire an ability to invade and metastasize due to the mutation of the genes that control cell growth and apoptosis.⁷ In addition to genetic predisposition, environmental influences such as traumatic irritation, tobacco, alcohol, nutritional factors, ultraviolet light, human papillomavirus and immunosuppression also plays an important role in

its development.⁸ Clinically Oral squamous cell carcinoma (OSCC) is staged by “tumor, node, metastasis” (TNM) classification system which is in line with the guidelines of American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC).⁹ Histopathologically Squamous cell carcinoma OSCC is graded as: Well Differentiated (Grade 1), Moderately Differentiated (Grade II), Poorly Differentiated and Undifferentiated squamous cell carcinoma (Grade III).¹⁰

Tumor suppressor genes and oncogenes are primarily responsible for genetic alterations at molecular level and thus are responsible for progression of normal epithelial cells to dysplastic squamous cells.¹¹ Various proliferative and apoptotic pathways are involved in normal cell cycle and growth.¹² When tumor suppressor genes get inactivated and oncogenes are activated these proliferative and apoptotic pathways are de-regulated which leads to tumor development. Genetic alterations in Ras-PI3Akt pathways are responsible for progression of normal epithelial cells to dysplastic squamous cells.¹² Some of the major genes involved in these pathways include kRas and Akt (Akt1, Akt2, Akt3). A number of studies have documented that Akt and k-Ras are the two major oncogenes responsible for cell growth, proliferation and metastasis thus causing development of OSCC.¹³

The Akt /PKB belongs to family of kinases which consists of three isoforms, i.e Protein Kinase Alpha (Akt1/PKB α), Protein Kinase Beta (Akt2/PKB β) and Protein Kinase Gamma (Akt3/PKB γ), They play important role in cell proliferation, cell survival and glucose metabolism. Akt was found to be highly expressed in OSCC¹⁴. The Akt/ PKB is a downstream effector of phosphatidylinositol 3-kinase (PI3K) and is frequently activated in human cancers, including OSCC.¹⁶ It is a key regulator of various cellular processes and promotes cell survival and proliferation. All three Akt isoforms have been associated with tumorigenesis and active mutations in Akt genes (Akt1, Akt2 and Akt3) and along with gene amplifications causes start of tumorigenesis. Thus, wide majority of HNSCC play active role in Akt signaling.¹⁵

KRas is also known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue. KRas gene acts as a molecular on/off switch and usually is in the “off” position. It normally turns on and causes cell growth in short bursts when it gets activated by the signal from growth receptor called EGFR. Once it is turned on recruits and activates proteins which effect growth factor and other receptors such as c-Raf and PI3-kinase.

KRas binds to GTP in the active state and possesses an intrinsic enzymatic activity which cleaves the terminal phosphate of the nucleotide converting it to GDP. Upon conversion of GTP to GDP, KRas is turned off. In this way, EGFR and KRas work together to control cell growth and development.¹⁶ To date, there has been only limited investigation of *KRas* mutations in HNSCCs. Of note, mutations in the *Ras* gene family (including *H*-, *K*-, and *N-Ras*) have been implicated in 30% of all human cancers. However, mutation frequencies within OSCCs vary from 5% to 50%.¹⁷ In countries like Pakistan people do not have much awareness about predisposing factors to oral cancers and understanding of oral lesions and they usually report too late when the cancer has reached to maximum grade and stage.¹⁸ Though studies emphasizing incidence, prevalence, pre-disposing factors and case reports have been published and public awareness programs at different forums and media campaigns have started, but genetic basis of these cancers which can ultimately help in curing the disease process have been minimally explored in Pakistan.¹⁹ Since genetic basis of Pakistani population and the pre-disposing factors are quite different from the countries in which the most of the molecular level has been carried out, so it is the time when we should start working on our own problems and then their management. This is the reason which let us to dig in the molecular study of oral squamous cell carcinoma. The aim of this study was thus to find out role of Akt and KRas in cancer progression in a sample of Pakistani population.

METHODOLOGY

Tissue specimens were collected from two public and three private hospitals. Those patients whose history, clinical / Radiographical examination made them susceptible for the cancer of the oral cavity were counselled for the biopsy. Biopsy was taken from the involved/ suspected sites of oral cavity with the proper written and informed consent. Biopsy of the 65 patients were then taken and Biopsy samples were fixed in formalin. After confirmation by the histopathological report 50 patients biopsy showed either well differentiated OSCC (Grade I) or poorly differentiated OSCC (Grade III), while the remaining biopsy reports showed moderately differentiated OSCC (Grade II). In order to establish the cascade we decided to study initial stages of cancer which is grade I and compared it with terminal stage of cancer which is Grade III. Three samples were also taken from normal individuals to identify the baseline gene and protein expression as a reference.

Genome Work: The Formalin Fixed Paraffin Embedded (FFPE) tissues blocks were then taken from the laboratory. These specimens were then processed for the genes analysis. First step was Total RNA Isolation which involved dewaxing of each sample. Each sample was then dried and stored at - 45°. Total RNA was then extracted with Mini Total mRNA isolation kit and quantified by using spectrophotometer method on ScanDrop™ using Winaspect software supplied with the instrument. The quantification was recorded for each sample (reading 260 nm / 280 nm). Total of 5 µL of sample was used to quantify each sample of RNA. To form DNA Reverse Transcriptase (RT) PCR Reaction was carried out. Primers were then designed using Light Upon EXtension (LUX™) primer design software. Real time PCR reactions were set up for each sample after RT reactions. All genes quantifications were statistically analyzed by SPSS.¹⁶

Statistical Analysis: Descriptive statistics was calculated for each variable in the study. One way ANOVA was used for determining the variance between genes. Tukey's was used for paired analysis between groups.

RESULTS

This study is based on fifty three (53) samples of which three were normal, twenty five (25) were identified histologically as grade 1 and twenty five (25) samples were identified histologically as grade III. Akt1, Akt2,

Akt3 and K Ras levels were assessed in each sample to see their expression in different grades.

In Tables 1-4 are recorded mean Ct values for Normal, Grade I and Grade III tissues of OSCC along with statistical analysis using one way anova. Table indicates the mean, Standard Error. The mean values were calculated from data of 53 samples. KRAS was analysed by Multiplex PCR with LUX primers.

DISCUSSION

Oral squamous cell carcinoma is a relatively common human cancer characterized by high morbidity, high mortality, and few therapeutic options. Genetic alterations related to the carcinogenesis or progression of oral squamous cell carcinoma is not properly understood. Though signaling pathways associated with the progression of oral squamous cell carcinoma have been established, yet the impact of age, ethnicity and environmental factors on the progression of oral squamous cell carcinoma have not been widely explored. Proper understanding of the cross-talks between these pathways and networks will form the basis of developing novel strategies for targeting multiple molecular components for more effective prevention and treatment of OSCC. This study, therefore, aimed at study the dynamics of Akt and KRas pathways in Pakistani (Punjab) population with the perspective of early & better understanding, diagnosis, treatment and prognosis of OSCC.

TABLE 1: EXPRESSION OF AKT1 IN IN WELL DIFFERENTIATED (GRADE I) AND POORLY DIFFERENTIATED (GRADE III) ORAL SQUAMOUS CELL CARCINOMA

Grades	Mean Ct values (1/ RFU)	Median Ct values (1/ RFU)	Std. Error	Range	P- value
Normal	24.03	23.99	0.23	23.66 - 24.44	p<0.01***
Well Differentiated Grade I	12.11	10.33	0.89	10.12 - 24.44	
Poorly Differentiated Grade III	6.46	5.60	1.06	2.00 - 24.00	

TABLE 2: EXPRESSION OF AKT 2 (MEANS) IN WELL DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE I AND POORLY DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE III

Grades	Mean Ct values (1/ RFU)	Median Ct values (1/ RFU)	Std. Error	Range	P- value
Normal	38.36	38.25	0.11	38.25 - 38.59	p<0.01***
Well Differentiated Grade I	12.11	10.33	0.78	24.00 - 38.02	
Poorly Differentiated Grade III	6.46	5.60	1.22	6.00 - 38.00	

TABLE 3: EXPRESSION OF AKT 3 (MEANS) IN WELL DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE I AND POORLY DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE III

Grades	Mean Ct values (1/ RFU)	Median Ct values (1/ RFU)	Std. Error	Range	P- value
Normal	42.00	42.00	0.58	41.00 - 43.00	p<0.01***
Well Differentiated Grade I	42.61	41.84	0.25	41.33 - 42.00	
Poorly Differentiated Grade III	18.37	16.33	1.55	15.03 - 43.00	

TABLE 4: EXPRESSION OF KRAS (MEANS) IN WELL DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE I AND POORLY DIFFERENTIATED ORAL SQUAMOUS CELL CARCINOMA GRADE III

Grades	Mean Ct values (1/ RFU)	Median Ct values (1/ RFU)	Std. Error	Range	P- value
Normal	35.88	35.66	0.22	35.66 - 36.33	p<0.01***
Well Differentiated Grade I	12.61	10.44	1.40	10.12 - 35.99	
Poorly Differentiated Grade III	7.77	7.36	0.18	6.33 - 8.99	

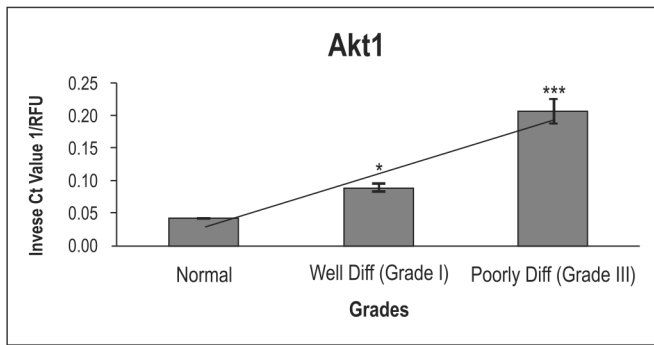


Fig 1

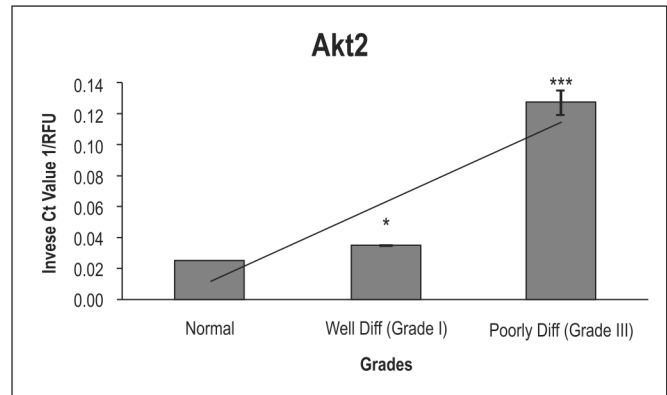


Fig 2

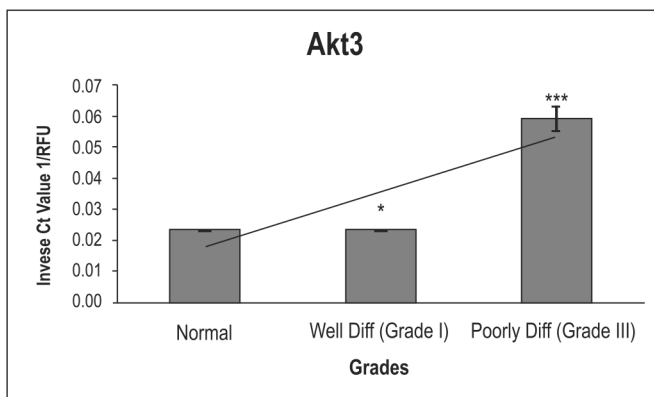


Fig 3

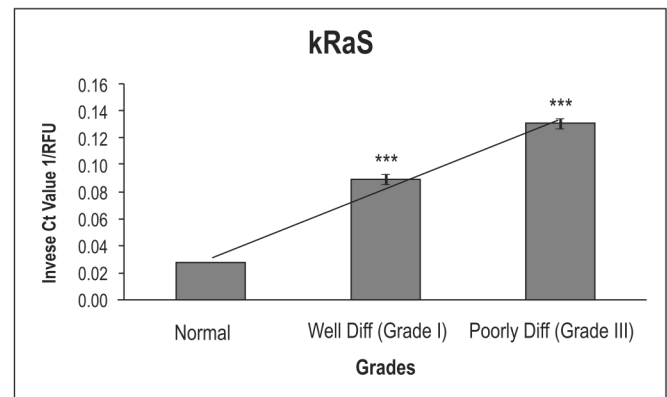


Fig 4

Fig 1-4. represents histograms of expression of isoforms of Akt and KRas in Normal, Grade I and Grade III tissues samples of OSCC. Presence of* on vertical bar is showing the significant/non-significant or highly significant value taken from the One way annova after applying the tukeys.

Our data demonstrate the involvement of three isoforms of Akt in two grades, namely, Grade I of well differentiated cells and Grade III of poorly differentiated cells. The data presented in this thesis is by enlarge, in accordance with those reported by earlier workers.²⁰ It has been shown by us that there is increased Akt expression in head and neck cancers (HNSCC) and its expression increases with the grades of OSCC.²¹ Another study concluded that Akt1 mutation contributes to initiation and progression of oral squamous cell carcinoma in both genders.²² Our data confirms this earlier report. Epithelial Mesenchymal Transformation (EMT) has been reported by a number of workers under the influence of Akt and that this is characterized by down regulation of the epithelial markers desmoplakin, E-cadherin, and beta-catenin, and up regulation of the mesenchymal marker vimentin.²³ Yet another study suggested that Akt inhibition could stop the Epithelial Mesenchymal Transition (EMT) thus a strategy involving Akt inhibition might be a useful therapeutic tool in controlling cancer dissemination.²⁴ Furthermore, it has also been observed that activation of Akt is a frequent event in human HNSCC because active Akt can be detected in these tumors with a pattern of expression and localization correlated with the progression of the lesions.²⁵ Although Akt 1, 2, and 3 share high sequence homology, clinical studies have suggested the existence of isoforms-specific roles of Akt in multiple human cancers.²⁶

In a recent review it has been illustrated that Akt signaling plays a dominant role in the progression of a variety of cancers, including, OSCC.²⁷ Furthermore, in a number of cancer cell lines Akt1 has been associated with tumor growth and cancer cell invasion. Akt2 seems to be related to survival, cell invasion and possibly metastasis. The role of Akt3 is by enlarge associated with metastasis and angiogenesis under the influence of Vascular Endothelial Growth Factors (VEGF).²⁸ In our study we have observed early rise of Akt1 in Grade 1 (2.5-fold). This is followed by further increase (3.5-fold) in Grade III. There is also a 0.5-fold increase of Akt2 in Grade I. However, Akt2 also increases by an order of magnitude to 4-fold in Grade III. As far as Akt3 is concerned, there is only marginal, insignificant increase in Grade 1 which rises appreciably in Grade III (3-fold). This dynamics of Akt forms, we believe is peculiar to OSCC.²⁹ These results are also in general agreement with those reported earlier in which increased levels of all forms of Akt: Akt1, Akt2, and Akt3 are shown to be elevated in increasing grades of oral cancer lesions.²⁷

This information clearly warrants the need for use of isoforms-specific Akt inhibitors in clinical management of oral cancer patients.

KRas, a protein within the *Ras* family, appears to be involved in signal transduction and cell cycle regulation. *Ras* gene family (including *H-*, *K-*, and *N-RAS*) has been implicated in upwards of 30% of all human cancers; however, mutation frequencies within OSCCs are varied (5-50%) and appear to be dependent on the specific RAS gene and interestingly, geographic location of the population studied.^{30,31} Accordingly it has been observed that in the western hemisphere there is a low frequency of mutations in Ras genes in head and neck carcinoma. Higher frequency has been found in countries such as India and Taiwan.³² In a study by Kuo et al, it has been concluded that people who developed betel nut chewing habits, in them, approximately 80% of all oral cancer deaths were associated with this habit.³³ In line with these results our study concludes that Kras activation may play a key role in the development of OSCC in betel and quid chewing in Pakistani Punjabi population. According to this it is suggested that Kras and Akt pathways given high priority in patients suffering from this cancer. Mutations in the BRAF, KRAS, and PIK3CA genes make at least a minor contribution to OSCC tumorigenesis, and pathway-specific therapies targeting these 2 pathways should be considered for treatment of OSCC in a subset of patients with these mutations. It is known that Ras genes are involved in a wide variety of human tumors.³⁴ Frequent Kras mutation have been reported in different neoplasms including OSCC.¹⁷ Our data on K Ras in OSCC are in agreement with these studies quoted vide supra, though its expression is more in the early stages of the tumor.

CONCLUSION

In conclusion, our study revealed that Akt and KRas activation is a significant prognostic indicator for OSCC and may provide a new method of treatment of OSCC. It is seen that Akt 1 expression rises to 2.5-fold in well differentiated tissues, the same rises continues to 3.5-fold in poorly differentiated tissues. The Akt-2 on the other hand shows only 0.5-fold increases from normal tissue in grade 1 tissues, but rises to 4-fold increase in grade-3 tissue. In the same tissues, there was no change in Akt-3 as compared to normal in grade I tissues yet, 3-fold increase has been recorded in grade III tissue. The oncogene K-RaS shows consistent increase of the order of 2.5-fold in grade-1 and 3-fold in grade-3.

REFERENCES

- 1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 56(2): 106-130.
- 2 Bhurgri Y, Bhurgri A, Usman A, Pervez S, Kayani N, Bashir I. Epidemiological review of head and neck cancers in Karachi. *Asian Pac J Cancer Prev*. 2006; 7(2): 195-200.
- 3 Parkin DM, Bray F, Ferley J, Pisani P. Global cancer statistics, 2002. *CA Cance J Clin* 2005; 55: 74.
- 4 Ali TS, Baig S. Evaluation of a cancer awareness campaign: experience with a selected population in Karachi. *Asian Pac J Can Pre*. 2006; 7(3): 391-5.
- 5 Chaudhry S, Khan AA, Mirza KM. Estimating the burden of head and neck cancers in the public health sector of Pakistan. *Asian Pac J Can Prev*. 2008; 9(3): 529-32.
- 6 Silverman S Jr. Demographics and occurrence of oral and pharyngeal cancers. The outcomes, the trends and the challenge. *J Am Dent Assoc*. 2001; 132 Suppl: 7s-11s.
- 7 Barbellido SA, Trapero JC, Sánchez JC, Miguel A, García P, Castaño NE, Martínez AB. Gene therapy in the management of oral cancer: Review of the literature. *Med Oral Patol Oral Cir Bucal*. 2008; 3(1): 15-21.
- 8 Mehrotra R, Yadav S. Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations. *Indian Journal of cancer*. 2006; 43(2): 60-66.
- 9 Monroe M, Meyers AD. Head and neck squamous cell carcinoma[Internet]. 2011[updated 2013, June 4th; cited 2013, June 4th]. Available from: <http://emedicine.medscape.com/article/1965430-overview>
- 10 Lindenblatt RC, Martinez GL, Silvia LE, Faria PS, Camisacca DR, Lourenco SQ. Oral Squamous cell carcinoma grading system –analysis of the best survival predictor. *J Oral OPathol Med*. 2012; 41(1): 34-9.
- 11 Pitiyage G, Tilakaratne WM, Tavassoli M, Warnakulasuriya S. Molecular markers in oral epithelial dysplasia: review. *J Oral Pathol Med*. 2009; 38(10): 737-52.
- 12 Choi S, Myers JN. Molecular Pathogenesis of Oral Squamous Cell Carcinoma: Implications for Therapy. *J dent res*. 2008; 87(1): 14-32.
- 13 Nakayama H, Ikebe T, Beppu M, Shirasuna K. High expression levels of nuclear factor kappaB, IkappaB kinase alpha and Akt kinase in squamous cell carcinoma of the oral cavity. *Cancer*. 2001; 92(12): 3037-44.
- 14 Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci*. 2001; 98(20): 10983-5.
- 15 Segrelles C, Moral M, Lara MF, Ruiz S, Santos M, Leis H. Molecular determinants of Akt-induced keratinocyte transformation. *Oncogene*. 2006; 25(8): 1174-1185.
- 16 Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nature Medicine*. 2004; 10 (8): 789-799.
- 17 Weber A, Langhanki L, Sommerer F, Markwarth A, Wittekind C, Tannapfel A. Mutations of the BRAF gene in squamous cell carcinoma of the head and neck. *Oncogene*. 2003; 22(30): 4757-4759.
- 18 Kazmi F, Iqbal S, Mumtaz M, Asad A. Awareness regarding screening of oral cancers in young dental graduates. *Pakistan Oral and Dental Journal*. 2012; 32(1): 56-61.
- 19 Mehboob B, Khan E, Khan E. Awareness about oral cancer among non medical university students of Peshawar. *PODJ*. 2011; 31(2): 392-395.
- 20 Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. *Nature Reviews Cancer*. 2002; 2(7): 489-501.
- 21 Crowell JA, Steele VE, Fay JR. Targeting the AKT protein kinase for cancerchemoprevention. *Mol Cancer Ther*. 2007; 6: 2139-2148.
- 22 Do H, Salemi R, Murone C, Mitchell PL, Dobrovic A. Rarity of AKT1 and AKT3 E17K mutations in squamous cell carcinoma of lung. *Cell Cycle*. 2010; 9(21): 4411-2.
- 23 Grille SJ, Bellacosa A, Upson J, Klein AJ, Van Rov F, Lee-Kwan W. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Research*. 2003; 63(9): 2172-2178.
- 24 Kang P, Svoboda KK. Epithelial-mesenchymal transformation during craniofacial development. *J. Dent. Res*. 2005; 84: 678-690.
- 25 Amornphimoltham P, Sriuranpong V, Patel V. Persistent Activation of the Akt Pathway in Head and Neck Squamous Cell Carcinoma: A Potential Target for UCN-01. *Clin Cancer Res*. 2004; 10 (12): 4029-4037.
- 26 Manning AD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*, 2007; 129(7): 1261-1274.
- 27 Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci*. 2001; 98(20): 10983-5.
- 28 Fortier AM, Asselin E, Cadrin M. Functional specificity of Akt isoforms in cancer progression. *Biomolecular Concepts*. 2011; 2(1): 155-158.
- 29 Moral M, Segrelles C, Lara MF et al., “Akt activation synergizes with Trp53 loss in oral epithelium to produce a novel mouse model for head and neck squamous cell carcinoma” *Cancer Research*. 2009; 69 (3): 1099-1108.
- 30 Downward J. Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer*. 2003; 3(1): 11-22.
- 31 Williams HK. Molecular pathogenesis of oral squamous carcinoma. *Mol Pathol*. 2000; 53(4): 165-172.
- 32 Ruiz-Godoy R LM, Garcia-Cuellar CM, Herrera Gonzale NE, Suchil BL. Mutational analysis of Kras and Ras Protein expression in larynx squamous cell carcinoma *J. Exp. Clin. Cancer Res*. 2006; 25(1): 73-78.
- 33 Kuo MY, J H Jeng JH, Chiang CP, Hahn LJ. Mutations of Ki-ras oncogene codon 12 in betel quid chewing-related human oral squamous cell carcinoma in Taiwan *Journal of oral pathology & medicine*: 2006; 23(2): 70-74.
- 34 Bruckman KC, Schönleben F, Qiu W, Woo VL, Su GH. Mutational analyses of the BRAF, KRAS, and PIK3CA genes in oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2010; 110 (5): 632-7.