

Dynamics of *Akt* Isoforms and Role of Immune Evader (*RCAS 1*) in Different Grades of Breast Cancer Tissues in Pakistani Women

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Abstract

Breast Cancer (BC) is the leading cause of death among females. It has been documented that *Akt* isoforms and kRaS control the proliferative pathways in most cancer including BC. To address the issue of breast cancer signaling through mRNA quantification along with proteins quantifications in *Akt* pathway by microbead assay.

A total of 42 specimens were selected from a pool of samples representing nearly all Bloom's Richardson's grades tissues (Normal, Hyperplasia, Ductal in situ Carcinoma, Grade I, Grade II and Grade III). No Grade IV sample was available. Total of 8 genes (*Akt 1*, *Akt 2*, *Akt 3*, *p53*, *RCAS 1*, *Bcl 2*, *BclxL*, and *Mcl 1*) and 11 proteins in PI3K / *Akt* - mTOR pathway were assayed simultaneously using microbead BioPlex™ assay. Results on quantification of *RCAS 1* gene (Immune Evader) indicates its significance by increased quantities to help to evade the breast cancer cells to distant places to metastasize in the body. Data obtained on *Akt* isoforms (*Akt 1*, *Akt 2*, *Akt 3*) show that all three play significant role in the progression of breast cancer. Our data indicate that in advance grades (grade II and III) *Akt 3* along with *Akt 1* act as oncogenes. Here we propose that *Akt1* and *Akt 3* appear to be involved in cellular growth/proliferation and angiogenesis/invasion respectively. Bioinformatics modeling have shown that the conformation of *Akt 3* differs from *Akt 1* and *Akt 2* in PH domain that interacts to surface membrane from inner side.

Based on our data, we conceived that roles of all three *Akt* isoforms in growth, hypertrophy, epithelial mesenchymal transition (EMT), adhesion, and metastasis been highlighted. As reported in other cancers, *Akt 3* appears to be involved in the phosphorylation of VEGF and Integrin family proteins, thereby facilitating invasion and metastasis in the aggressive stages of breast cancer. We propose a model with dynamics of *Akt* and *RCAS 1* for metastasis.

Introduction

In this part of world (Pakistan), breast cancer is the second most common cancer after lung cancer and is ranked first in women^[1-4]. The current information indicates that breast cancer is most common in young women of Pakistan^[5-9]. It has been substantiated that *Akt* and kRaS-MAPK are dominant proliferative pathways stimulated by activated Receptor Tyrosine kinase (RTK)^[10,11]. *Akt* (also termed Protein Kinase B – PKB) works as double edged sword for cell survival as well as inhibitor of apoptosis^[12,13]. It has been identified that *Akt*, especially *Akt 1* isoform is the principle oncogene^[7]. More recently, three isoforms namely, *Akt 1*, *Akt 2*, *Akt 3* have been reported^[1,2]. The function of each isoform has yet to be fully substantiated. So far three corresponding *Akt* genes (*Akt 1*, *Akt 2* and *Akt 3*) have been reported^[14-17]. The differences in functional abnormalities observed in *Akt* null mice suggest that all isoforms have physiologically diverse roles^[18]. Characterization of *Akt 1* (Ch 14q32.33), the prototype isoform, makes it the key signaling molecule in diverse cellular processes including muscle hypertrophy^[19-21] and presented as a principle proliferative molecule in many types of cancer^[22,23]. *Akt 2* (Ch 19q13.2) in contrast to *Akt 1*, knockout

results in mild growth deficiency with severe diabetic phenotype (insulin resistance)^[23,24]. The function of *Akt 3* (Ch 1q44), a comparatively less explored isoform, is predominantly associated with brain growth and testes^[20,25,26]. The *Akt* isoforms are ~480 amino acids long protein with a large number of posttranslational phosphorylation sites^[10,27]. Initial records indicate that *Akt 3* is consistently detected in cancer tissues as compared to normal tissues. However, this remains controversial^[28,29]. Furthermore, increase in quantities of *RCAS 1* seems to be associated with release of *Akt 3* from Golgi complex^[30-32]. *RCAS 1* is over ex-

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pressed in many patients suffering from breast carcinoma and its expression levels correlate with tumor grades as well, suggesting that it may be involved in immune escape^[31,33]. *RCAS 1* is also been implicated in regulatory functions with VEGF in ovarian cell line^[34,35].

Bcl 2 family genes / proteins maintain a critical balance between cell proliferation and apoptosis^[36,37]. These antiapoptotic proteins include *Bcl 2*, *BclxL* and *Mcl 1*. These proteins have a common hydrophobic groove formed by BH 1, BH 2 and BH 3 domains^[38-40]. Evidence is also available for the overexpression of some members of the *Bcl 2* family in breast cancer^[36,40]. The tumorigenic potential of these proteins has been well documented in animal models in which expression of *Bcl 2* oncogene has been reported in a variety of tumors and in lymphomas^[41,42]. High expression of *Bcl 2* has been observed in Estrogen Receptor (ER) and Progesterone Receptor (PR) positive breast cancer^[43,44]. *BclxL* on the other hand is related to the invasion and metastasis of some solid tumors^[40,45,46]. *Mcl 1* is shown to be regulated by mTOR complex for transcription and translation^[47,48]. Significance of *Mcl 1* expression has been studied in a number of tumor types. It is linked to poor patient prognosis and survival^[49].

The three isoforms have been studied in melanoma^[14,50,51]. In melanoma there is sharp rise in *Akt 3*^[52,53]. This aspect has, however, not been studied adequately in other cancers, including breast cancer. Addition to it, little information is available on the molecular pathogenesis of *Akt 1*, *Akt 2* and *Akt 3* in various grades of cancer. Literature doesn't provide concurrent role of the three isoforms in breast cancer. Lack of this information about the dynamics of *Akt* isoforms has prompted us to examine the amplification of *Akt 1*, *Akt 2* and *Akt 3* in normal, Grade I, Grade II and Grade III tissues supported by assay of 11 phosphoproteins of *Akt* pathway. This will be first report on the dynamics of *Akt* isoforms and their associated substrates in Pakistani (Punjabi) population.

Materials and Methods

Specimen Collection and Processing

Total of 42 breast cancer specimens in different Bloom Richardson's grades^[28] were collected from three major public and private hospitals of Lahore, Pakistan. The specimens were stored at -40°C (Forma Scientific, USA) that includes Formalin Fixed Paraffin Embedded (FFPE) tissue blocks and Formalin Fixed (FF) tissues. The tissues were grounded to powder form in thistle and mortar with liquid nitrogen (Fine Gas (Pvt) Ltd, Lahore). Materials were taken out when ever needed on crushed ice bucket (Zellegra Eismaschinen, Germany) for further processing.

RNA Isolation, cDNA, Primer Design and qPCR Quantification

Total RNA was extracted by LiCl – Urea method (3M LiCl, 6M Urea, 50 mM Tris (7.4), 1 mM EDTA Na₂, 0.5% Sarkosyl (Serva, Germany) to preweighed tissue specimen (40-60 mg)^[54]. Total of 15 – 20 μL of 10% SDS was added to LiCl – Urea reagent to enhance homogenization with Micropestle (Bel Air Products, USA) two times for 5 minutes each. Total RNA was extracted from cells treated with Proteinase K (Vivantis, Malay-

sia) in PK buffer (10 mM Tris (8.0), 2 mM EDTA Na₂ (8.0), 200 mM NaCl, 0.5% SDS and 200 $\mu\text{g} / \text{mL}$ Proteinase K (20 mg/mL) for 30 minutes at 37°C . Samples were Phenol – Chloroform – Isoamyl alcohol (PCI) extracted (25:24:1) (MP Bio, USA) twice and with chloroform (Merck, Germany) once^[55]. The aqueous phase was precipitated with HPLC grade 2-Propanol (BDH, UK) over night at -40°C ^[56]. A second precipitation was done with $\frac{1}{2}$ volume Ammonium acetate (5 M) and 2.5 volume of molecular grade ethanol (Merck, Germany) after pellet been dissolved in 100 μL of RNase free water. Sample was centrifuged (Sigma, Germany) at 17 kg for 20 minutes at 4°C , pellets washed with 70% RNase free ethanol, air dried and then at 45°C for 10 minutes on heating block (T-box, Jena Analytika, Germany).

Pellets were dissolved in molecular grade water and RNA concentration was adjusted to 1 $\mu\text{g}/\mu\text{L}$ (Jena Analytika, Germany) used for cDNA synthesis by setting up a RT reaction with Maxime™ RT Random Hexamer kit (Intron Biotechnology, S. Korea) in 20 μL volumes. The RT PCR program was written on Thermocycler (BioRad C1000 with CFX96 detector, USA) with annealing temperature of primers at 16°C (10 minutes), 25°C (10 minutes), 37°C (40 minutes) and final elongation at 42°C for 20 minutes. Reaction was denatured at 95°C for 5 minutes. Multiplex real time (rt) quantification were done with gene specific primers for *Akt 1* (AlexaFluor 647), *Akt 2* (JOE), *Akt 3* (Cy 5), *p53* (FAM) with GAPDH (ROX) as housekeeping normalization. Primers were designed from FASTA sequences uploaded on to either Light Upon eXtension (LUX™) primer design software; D – LUX primer design software (Invitrogen Corp., USA) or QuantPrime (Max Planck Institute, Germany). Labeled primers with fluorescent tags were synthesized from BioBasicInc, USA or IDT, USA. Primer sequences are given Table 1 and Table 2. All qPCRs were done by using qMaster Mix with UDG (Invitrogen, USA). Total of 2 μL cDNA reaction, 400 nmol of each fluorescent labeled primer were used in each 20 μL reactions. Amplification protocol was initiated with heating at 52°C (2 minutes), denaturation at 95°C (10 minutes), cycle denaturation at 95°C (20 seconds), annealing at 58°C (30 seconds), and elongation at 72°C (30 seconds). The fluorescent signal was collected for 45 cycles. The CFX manager (BioRad, USA) was used to optimize the quantified data. Each quantification reaction was done three times in separate experiments.

Micro Bead Milliplex MAP 11-plex Assay

Akt / mTOR Panel (Milliplex, USA) was selected for 19 selected samples with traditional Bioplex / Luminex MAP based platform to detect simultaneously multiple protein quantities. Each sample was weighed ~ 50 mg in sterile, oven dried microfuge tube for protein isolation. Assay was run according to the manufacturer manual's protocol provided with the *Akt – mTOR* panel kit. Before the processing of each sample, tissue powder was washed in 0.5 mL ice cold TBS and to pellet 150 μL ice cold 1X Milliplex MAP Lysis Buffer containing freshly prepared 1X protease inhibitors and 1X phospho inhibitors cocktails (Serva, Germany). Gently rock the lysate for 10 - 15 minutes at 4°C . Remove particulate matter by high speed centrifugation at 15k g. Aliquot clarified supernatant and store the lysate at -40°C . Lysate protein concentrations were determined by diluting 1:10 in PBS by Qubit™ (Invitrogen, USA) protein assay.

Statistical Data Analysis

All genes and protein quantifications were statistically analyzed by SPSS 17 (SPSS, Inc. USA) using various tools in the software.

Bioinformatics Tools: Different bioinformatics tools were used to get 3D protein structures of different molecules which includes; *Akt 1*, *Akt 2*, *Akt 3* by loading protein FASTA Sequences on to LOMETS software (<http://zhanglab.ccmh.med.umich.edu/LOMETS>). The molecules were displayed by PyMol, Version 1.3 (2010).

Results

Physical features of isoforms

In the Figure 1a that represents the conserved domains of the three isoforms. The same figure also shows the percentage of homology between three *Akt* isoforms. Pleckstrin Homology (PH) domain has the highest homology percentage between the three isoforms^[29]. The highest percentage of homology is observed between *Akt 1* and *Akt 3* (84%), followed by 80% between *Akt 1* and *Akt 2*. There is only 76% homology between *Akt 2* and *Akt 3*. Variations in other domains have also been recorded (Figure 1a).



The primary sequence was down loaded in FASTA format and protein alignment was done with MAFFT tool. Serine, threonine and tyrosine amino acids were highlighted with; S Serine T Threonine Y Tyrosine in the aligned amino acid sequence. The box represents a hyper variable region in the kinase-regulatory region where Y⁴⁵² is identified as unique addition. These amino acids are labeled in the LOMETS 3D prediction software. All 3D data is visualized in PyMol. The highlighted box represents the variable region in regulatory linker region of the three Akt isoforms

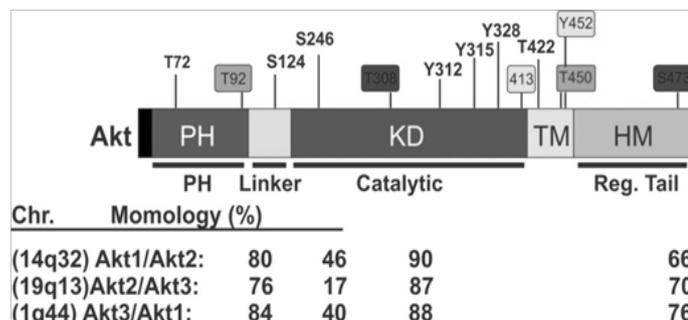


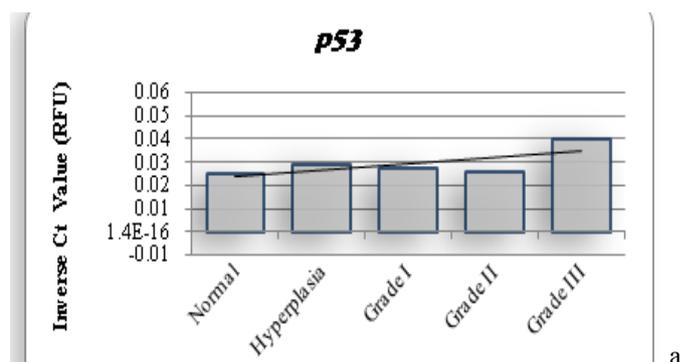
Figure 1a: Clustal format | Fasta format | MAFFT result.

Figure 1b shows the amino acid alignment of three isoforms (www.ncbi.org). It has been observed that there are major variations in amino acid sequences of the three isoforms that

reside in their regulatory domain. Figure 2 shows the comparison of tertiary structure of three isoforms (<http://zhanglab.ccmh.med.umich.edu/LOMETS>). It may be seen, as expected from the major homologies between three isoforms that there is very little difference in their tertiary structure. *Akt 3*, however, shows an open loop in which Y⁴⁵² is available for phosphorylation. The significance of this difference is discussed elsewhere in this report. It is now known that PIP 3 mediated phosphorylation of Akts by PDK 1 in kinase domains of *Akt 1* - T³⁰⁸, *Akt 2* - T³⁰⁶, and *Akt 3* - T³⁰⁵ and by PDK 2 in the regulatory domains of *Akt 1*-S⁴⁷², *Akt 2*-S⁴⁷⁸, and *Akt 3*-S⁴⁷⁴ [57]. It is also known that the phosphorylated isoforms are negatively regulated by PHILPP1 and PHLiPP2. Phosphorylated threonine in the catalytic domain in each isoform is deregulated by SHiPs^[58-60]. In figure 3 (a, b, c, d) are recorded results of amplification of *Akt* isoforms in the normal, Grade I, Grade II, and Grade III tissues of breast cancer. It may be noted that *Akt 3* is conspicuously absent in the normal tissue while *Akt 1* and *Akt 2* show normal values though *Akt 2* is higher than *Akt 1*. Progressively *Akt 1* shows significant increase through Grade I, Grade II and Grade III. There is eye-catching-difference in *Akt 1* in grade I and Grade III. *Akt 3* shows highly significant increase in Grade III compared to Grade I and Grade II. It shows elevation from the normal in Grade I and Grade III. The dynamics of three isoforms obtained in the study indicate that there is a considerable variation in three isoforms during the early and later stages in breast cancer tissue. A few samples of DISC and hyperplasia were also examined (data not shown in this report). From the data obtained on *Akt* isoforms the possible conceived role of three isoforms is presented in figure 4. In normal tissue only *Akt 1* and *Akt 2* are present. However, in the progression of breast cancer, there is a major event of transformation of epithelial cells to mesenchymal cells. For this at least in breast cancer we have observed that all three isoforms play a positive role in this transformation. As the tumor progresses, further invasive and metastasis stages, *Akt 1* and *Akt 3* seem to play dominant roles in metabolism, protein synthesis, proliferation, adhesion and growth^[61,62].



Figure 1b: Structure and *Akt* isoform Domains. PH – Pleckstrin Homology Domain, KD – Kinase Domain, TM – Trans membrane region, HM – Hydrophobic Motif (Regulatory tail). S – Serine, T – Threonine, Y – Tyrosine (Modified from Liao and Hung, 2010).



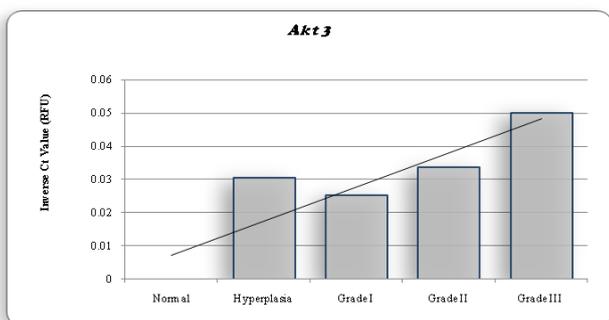
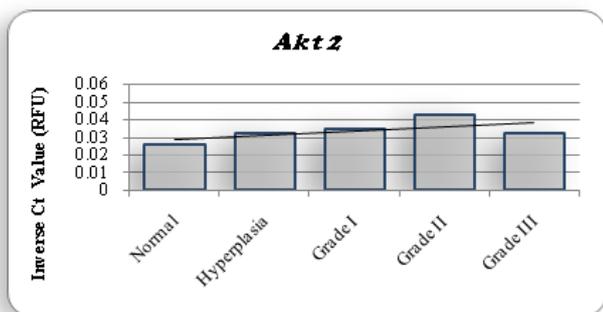
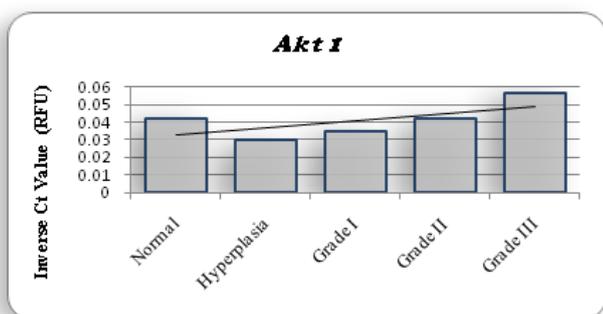


Figure 2

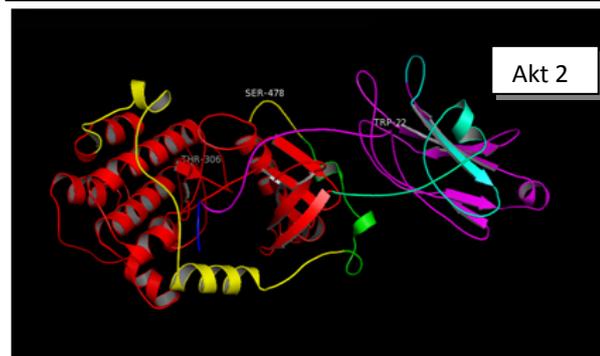


Figure 3: The three *Akt* Isoforms are presented the 3D structures of *Akt 1*, *Akt 2* and *Akt 3*. After downloading the sequence of each isoform from NCBI site, gene accession number NM_005163.2 (*Akt 1*), NM_001626.3 (*Akt 2*), NM_181690.1 (*Akt3*). This was done to identify the possible phosphorylation sites in each isoform (www.phosida.org). It's known that *Akt 1*, 2 and 3 are phosphorylated at threonine 308, 307 and 305; at serine 474,475 and 472. These Threonine sites are phosphorylated by PDK 1. However serine sites are phosphorylated by mTOR C2, PDK 2 and ILK. These sites as may be seen in the 3D structure are exposed at the surface and are available for phosphorylation by the respective enzymes. In *Akt 3* however additional sites: Ser⁴¹³, Y⁴⁵² is also available for phosphorylation in the regulatory domain. The possible significance of these two sites has not been reported.

b

c

d

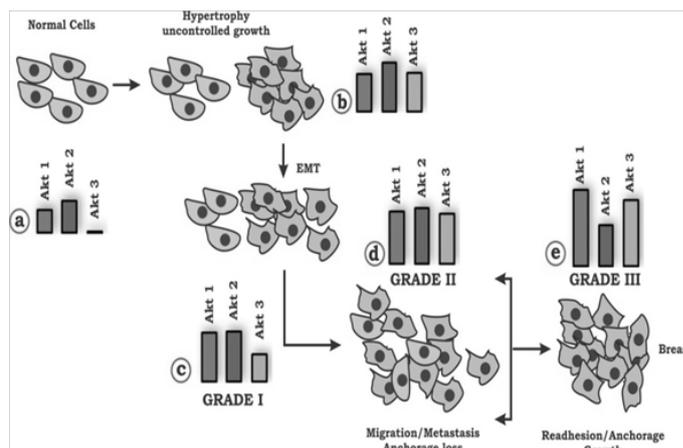


Figure 4: The figure demonstrates the possible conceived role played by three isoforms of *Akt* that is *Akt 1*, *Akt 2* and *Akt 3*. It may be seen that in Normal tissue only *Akt 1* and *Akt 2* are present. However, during the progression of breast cancer, there is a major event of transformation of epithelial cells to mesenchymal cells. For this at least in breast cancer we have observed that all three isoforms play a positive role in this transformation. As the tumor progresses further into invasive and metastasis stages *Akt 1* and *Akt 3* seem to play dominant roles in metabolism, protein synthesis, proliferation, adhesion and growth.

Like the genes, 11 proteins associated with *Akt*-mTOR pathway were estimated in normal and each grade of breast cancer tissues. The measurement of 11 phosphoproteins was simultaneously accomplished with Bioplex / Luminex MAP based platform as described under materials and methods. The quantification was undertaken to find out whether these could serve as possible specific markers for various stages of breast cancer^[63]. These proteins are mostly associated with metabolic requirements of proliferating cells. The estimated proteins included *Akt* (S⁴⁷³), mTOR (S²⁴⁴⁸), GSK3 α (S²¹), GSK3 β (S⁹), p70 S6K (T⁴¹²), RP S6 (S^{235/S236}), IRS 1 (S³¹²), PTEN (S³⁸⁰), TSC 2 (S⁹³⁹), IR (Y^{1162/Y1163}), and IGF 1R (Y¹¹³⁵ / Y¹¹³⁶). The data obtained are recorded in table 1.

Table 1: Table represents values of various proteins ng/mL in normal and different Grades of breast cancer tissue.

S. No	Names of Proteins	Normal	Hyperplasia	Grade I	Grade II	Grade III
1	pAkt (S ⁴⁷³)	19.08	25.81	24.50	23.00	19.75
2	pmTOR (S ²⁴⁴⁸)	19.08	20.66	20.08	18.25	18.50
3	pGSK 3 α (S ²¹)	14.58	16.47	15.42	18.42	16.25
4	pGSK 3 β (S ⁹)	9.42	12.00	12.25	10.75	7.25
5	pp70S 6K (S ⁴¹²)	37.83	21.09	41.33	38.00	40.25
6	pRPS6 (S ^{335/336})	17.67	18.97	21.50	35.75	14.00
7	pPTEN (S ³⁸⁰)	281.08	291.78	294.17	278.58	281.25
8	pTSC2 (S ⁹³⁹)	15.00	23.84	19.69	16.50	24.50
9	pIRS1 (S ³¹²)	8.24	12.81	10.75	11.58	12.00
10	pIR (Y ^{1162/1163})	24.67	29.41	23.50	26.08	24.50
11	pIGF 1R (Y ^{1135/1136})	10.58	11.72	15.58	12.75	13.00

The dynamics of RCAS 1 (Receptor associated Carcinoma Antigen on SiSo cells) like Akt isoforms was assayed in normal Grade I, Grade II and Grade III breast cancer tissues. RCAS 1 is strong indicator of immune evasion^[31,34]. In all grades the relative amplification compared to normal tissue has been observed: more so in Grade II and Grade III. This indicates positive role of it in immune evasion, especially in the aggressive tissue. There is no significant difference in the amplification of any antiapoptotic genes (*Bcl 2*, *BclxL* and *Mcl 1*). This indicates that anti apoptotic role of these proteins remain unchanged. High levels of any of these *Bcl 2* family proteins in Grade I, Grade II and Grade III tissues can serve as a major marker in prognosis of breast cancer after chemotherapy.

Discussion

In cancer, a large number of genes are known to be de-regulated^[64,66]. These include the genes of cellular proliferative RaS/MAPK pathway and survival Akt pathway^[66]. Several lines of evidence suggest that hyperactivation of Akt signaling along with RaS transform normal cells to cancerous cells^[67-69]. In view of these findings the present study was undertaken to obtain data on the dynamics of Akt isoforms and eleven proteins associated to Akt pathway in progression of breast cancer in various stages. In addition, three antiapoptotic genes namely *Bcl 2*, *BclxL* and *Mcl 1* were quantified to assess their contribution and role in cellular proliferation. The three isoforms of Akt have shown close similarities in three dimensional structures as shown in figure 2. In spite of these similarities, specific functions have been assigned to each isoform especially in their modulations during the early and later stages of breast cancer^[17,70]. Like others, we have also observed similar distribution of the three isoforms in normal, grade I, grade II and grade III breast cancer tissues. For instance, Akt 3 isoform is conspicuously absent in the normal tissue but is highly elevated in grade III tissues. This is in agreement with those reported by others^[14,16,21,71-73]. Some recent reviews have discussed the functional specificity of Akt isoforms^[2,74-75]. For instance, it has been suggested that Akt isoforms have relative importance in altered cells. It is substantiated that Akt 1 or Akt 2 or Akt 3 deficient mice, though viable, yet they have several deficiencies^[76,77]. Another study Akt 2 knockout mice exhibited

disorder in glucose metabolism (diabetes)^[78,79]. This is accompanied by insulin resistance^[80]. In Akt 3 deficient mice the brain size is reduced^[16,81,82]. It has also been reported that Akt 2 levels are higher in 25% of breast carcinoma^[24,83]. Compared to this, Akt 3 isoform is highly elevated in advanced stages of melanoma^[71,72,84]. Furthermore, high level of Akt 3 activity is demonstrated in estrogen deficient breast cancer^[35,85,86]. In essence, we have concluded from our data that differential constitutive elevation of Akt isoforms is responsible for initiation as well as progression of breast cancer as reported in other cancers. These results are in agreement with those reported by others^[68,71,72,83].

A closer examination of our data reveals a relative progressive increase in Akt1, Akt 2 and Akt 3 in various grades of breast cancer tissue. This has been summarized in Normal, Grades I,II and III tissues (Figure 3). It has been documented that Akt 1 is a major isoform involved in proliferation of cancerous tissues^[83,87,88]. Accordingly, the dominance of Akt 1 observed in this study is not surprising. This is further fortified by recent experimental evidence in which Akt 1 was down regulated by siRNA. This resulted in reduced proliferation^[89,90]. In the same experiment, falls in the levels of Akt 1 resulted in reduced expression of Cyclin D1^[85,91,92]. However, evidence to the contrary also exists. For instance, for purposes of proliferation, the role of Akt 2 cannot be minimized^[79,87-95]. This was shown by enhancing the levels of Akt 2 in absence of Akt 1. In this case the proliferation was restored to the original rate of growth. It has been demonstrated that both Akt 1 and Akt 2 were required for *in vitro* and *in vivo* growth of human colon cancer cell line HCT 116 and DLD 1^[96].

In view of our data which show high levels of Akt 1 and Akt 3 and reduced levels of Akt 2 in aggressive Grade III tissue, we are inclined to interpret that these changes are related to increase in mass and metastatic transformation which is characteristic of this stage^[8]. In an excellent review by Sheng *et al*^[15], it has been argued that metastasis must meet a minimum of nine requirements which include: relaxed proliferation, reduction in adhesion, a change in interaction with Extra Cellular Matrix (ECM), rapid turnover of altered fibroblasts, increase in the demand for nutrients and blood supply (angiogenesis), and focus of proliferation on metastatic sites followed by implantation^[97,98]. It appears that in our case, based on quantitative gene analysis of 42 samples, the above conditions of metastasis are fulfilled primarily by Akt 1 and Akt 3 isoforms^[99].

Though our data properly exhibits the dynamics of Akt isoforms in various grades of breast cancer tissue, yet it has to be explained whether in our case the increase of mRNA is due to; a) increased turnover of mRNA, b) genetic modifications, or c) gene amplification. It has been reported that genetic modifications are very rare in Akt^[16]. Similarly, gene amplification of Akt has not been reported in breast cancer^[20,100]. The differential elevation of mRNA of three isoforms leads to the conclusion that mRNA of each isoform is separately regulated. Furthermore, we have examined conformational differences in the three Akt isoforms using bioinformatics tools described under materials and methods. Interestingly enough, it has been reported that transplantation of PH domain of Akt 2 onto Akt 1 didn't restore the function of proliferation characteristic of Akt 1^[76,94]. We were particularly interested in identifying differences in 3D structure of PH domain for each isoform along with its phosphorylation

sites^[101,102]. 3D structures of the three isoforms, shown in figure 2, have been constructed using LOMETS (<http://zhanglab.cmb.med.umich.edu/LOMETS>). It may be seen in Figure 2 that in addition to putative phosphorylation sites in the three isoforms, additional phosphorylation sites are available in *Akt 3*. The new sites of phosphorylation are S⁴¹³ and Y⁴⁵². Whether these sites are actually phosphorylated in the signaling process remains to be determined. It is interesting to note, as is evident from our data that in Grade III along with *Akt 1* and *Akt 3*, *kRaS* is also elevated. This situation has been reported in literature in various types of cancer^[35,103,104]. A model of *Akt* isoform dynamics has been reported earlier. The model is based on the modulation of only *Akt 1* and *Akt 2*. In this report the model presents elevated *Akt1* with depressed *Akt 2*. In EMT the situation is reversed and remains so in the metastatic stage^[76,105]. In the data we are reporting in breast cancer patients in Pakistani females, we have observed the elevation of *Akt 1* and *Akt 3* in Grade III tissues. It is, however, important to point out that *Akt 3* is not detected in the normal tissue (Figure 3). On these bases we can suggest that elevation in *Akt 1* and *Akt 3* in Grade III tissues can be taken as biomarkers for aggressive stages of breast cancer. This calls for use of specific inhibitors of these two Akt isoforms for the treatment of cancer^[27,106].

In the Pakistani population we are reporting for the first time that *Akt 3* isoform along with *kRaS* play a dominant role in the aggressive stage of breast cancer. It is also of interest that in this population *Akt 2* is the first isoform to be elevated in hyperplasia. This is followed by elevation of *Akt 1*, *Akt 2* and *Akt 3* in subsequent stages (Figure 3 a, 3b, 3c).

In view of the well-known immunosuppressive role of *RCAS 1*^[33,107-109] we were interested to study modulations of *RCAS 1* because of its relationship with *Akt 3* in various grades of breast cancer tissue. It has been documented that in tumor cells *RCAS 1* expression plays an important role in evading the cancer cells from the immune system surveillance. This promotes progression, invasion and metastasis^[27,34]. The antigen has been used as a prognostic marker in Normal, Hyperplastic and malignant uterine endometrium^[3,109]. It has been identified as a possible factor in endocrine-immune interaction in breast cancer^[33,110]. Elevated levels of this antigen have been reported in hepatocellular carcinoma^[31], gastrointestinal tract, non-small cell lung carcinoma, gall bladder, breast, ovarian, endometrial and cervical cancers^[33,111]. In our data presented in Figures 3f show the increase of *RCAS 1* in Hyperplasia, Grade I, Grade II and Grade III tissues. This increase is directly related to high levels of *Akt 3*^[84]. The antigen is also listed as EBAG 9 is known to bind estrogen receptor^[112] thus activation of estrogen binding receptor. Gene suppression at transcription level and of immune system with MHC class 1 molecule on surface of tumor cells signifies various mechanisms that are in place in the cell^[34,113]. In view of this information it appears that elevated levels of *RCAS 1* in company with *Akt 3* promote immune cell evasion, promotes survival, boost invasion and metastasis^[108,114].

Similar conclusion could also be drawn with references to the levels of anti-apoptotic genes: *Bcl 2*, *BclxL* and *Mcl 1* (Figures 3g, 3h, 3i). Our data also indicate that quantification of *Bcl 2* family genes along with *RCAS 1* could also be used as good prognostic markers as seen in prostate cancer^[40,115,116]. Such a prognostic study must be accompanied by determining the lev-

els of *Akt 3* and anti apoptotic genes in the same tissue. This relationship of *RCAS 1* with antiapoptotic genes and *Akt 3* is being reported for the first time in Pakistani (Punjabi) population.

The proteins that were assayed for normal and different grades of breast cancer tissues are listed in table 1. Little information is available about the modulations of *Akt* and its substrate proteins in various grades of breast cancer. In our hands *pAkt*, *pGSK 3β*, *pTSC 2*, *pIRS 1* and *pIR* can be conveniently used as biomarkers for Grade I and Grade II. However, we propose that in Grade III, *pTSC 2*, *pIRS 1* and *pIGF 1R* quantification may serve as useful biomarkers. The proteins which are depressed in this grade include *pGSK 3β* and *pRPS6*. Comparable analytical data on these proteins in breast cancer tissue on various grades is not available in literature and are being reported for the first time. This could promote the process of grading breast cancer combined with other pathological information on Bloom-Richardson system^[28]. It is of great interest to note that simultaneous analysis of gene reported in this study along with protein analysis could be of help in selecting future inhibitors for *Akt* and its substrates. The observation that levels of *pAkt* as well as *pGSK 3α*, *pTSC 2*, *pIRS 1* and *pIGF 1R* are lowest in normal tissues (Table 1) may be of help in comparing of these proteins with their levels in various grades of cancerous tissues. *pPTEN* does not show any variation in the normal tissue as compared to its values in all other grades of cancer. *pPTEN* in active form dephosphorylates the PIP 3K stimulated phosphorylation of inositol at carbon 3^[117,118]. The sustained levels of *pPTEN* in all tissue grades indicate its ineffectiveness to dephosphorylate PIP 3. We are therefore; suggest that elevated *PTEN* in the normal tissue is a mutated form of this gene (Data not shown). This has been previously reported in several studies^[58,63,119-121].

The quantification of the proteins reported here, as expected, provided interesting information with regard to *pIRS 1* and *pIGF 1R*. Insulin is the ligand for the two receptors. These two proteins are elevated in all grades of breast cancer tissue^[122]. This is indicative of increased glucose metabolism (energy) as is required during growth, proliferation and metastasis^[23]. It has been previously reported that *GSK 3α* and *GSK 3β* were upregulated in normal prostate and in other cancers^[123]. However, in breast cancer *GSK 3β* increases in Grade I and both *GSK 3α* and *GSK 3β* increase in Grade II. This is again indicative of the availability of energy through glucose metabolism^[67,68,124].

We have estimated total mTOR complex phosphorylated at S²⁴⁸. mTOR C2 phosphorylates *Akt* at S⁴⁷² after its activation by *pRheb*^[79,106,110]. Other substrates of mTOR complex include *STAT3*, *S6K1 (p70)*, *4E-BP1* and *ULK1/ATG13*^[94,125]. In this way it promotes growth, proliferation, survival, angiogenesis and metabolic activities in various cancers^[126,127]. Like others, we have observed increased levels of phospho p70 S6 kinase and *pRPS6*. Alternatively, *RPS6* is also phosphorylated by *p90 (RSK)*. In that case it inhibits the selective transcription of various other genes^[94,128] making significant changes in normal cell signaling cascades. This is more evident from phosphorylated *RPS6* that promotes growth factors and tumor promoting agents^[128].

Conclusion

In essence, our genomic and proteomic data provide substantial evidence that *Akt 1* and *Akt 3* provide signals for proliferation of tumor (size) and invasion – angiogenesis. Furthermore, the elevated levels of pIR, pIGF 1R, pIRS 1 in glucose metabolism accompanied by phosphorylation of GSK 3 α and GSK 3 β provide higher energy source for growth, survival, proliferation and angiogenesis. The subtle dynamics of genes and proteins reported in this study may be of some help in selection of therapeutic molecules for breast cancer.

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