SPANDIDOS PUBLICATIONS

Overexpression of phospho-Akt correlates with phosphorylation of EGF receptor, FKHR and BAD in nasopharyngeal carcinoma

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Abstract. The Akt pathway is one of the most common molecular alterations in various human malignancies. However, its involvement in nasopharyngeal carcinoma (NPC) tumorigenesis has not been well established. In this study, the status of Akt activation and expression of its upstream and downstream molecules was investigated in 64 NPC and 38 non-malignant nasopharyngeal tissues by immunohistochemistry. The hotspot mutations of *PIK3CA*, encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), were also determined in 25 of these NPC tissues. No hotspot mutations were found in any of the samples tested. Akt was activated in 27 (42.2%) and 23 (35.9%) NPCs, as indicated by p-Akt (Thr308) and p-Akt (Ser473) immunoreactivity, respectively. PTEN loss did not correlate statistically with activated Akt. However, a positive correlation was observed between activated Akt and phospho-epidermal growth factor receptor (p-EGFR), suggesting that the EGFR signaling might be one of the upstream regulators of the Akt pathway. The phosphorylation of forkhead (FKHR) and Bcl-2 associated death domain (BAD), but not mamalian target of rapamycin and glycogen synthase kinase-3ß, was significantly correlated with Akt activation. This implies that Akt promotes cell proliferation (as estimated by Ki-67) and survival, at least, through the inactivation of FKHR and BAD in NPC. Our data revealed that the EGFR/PI3K/Akt signaling pathway is important in NPC pathogenesis and that *PIK3CA* hotspot mutations are rare in NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is one of the most frequently occurring malignant tumors of the head and neck. One of the most unique features, which make this cancer a special entity by itself, is its geographical and population distribution. While NPC exhibits a high incidence in Southern China and parts of Southeast Asia, it is less common in the United States and Western Europe. Epidemiologic investigations suggest that the etiology of NPC is associated with a complex interaction of genetic, viral, environmental and dietary factors. The molecular characterization of cellular signaling may provide a better understanding of the disease etiology and pathogenesis.

Overexpression of the epidermal growth factor receptor (EGFR) is often found in NPC, suggesting that its signaling may play a significant role in the NPC pathogenesis (1,2). In conveying the downstream signaling effects of EGFR, the Akt (protein kinase B) has emerged as a central player through the activation of phosphatidylinositol 3-kinase (PI3K) (3). Activated PI3K catalyzes the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3) at the cell membrane. PIP_3 is essential for the translocation of Akt to the plasma membrane where it is phosphorylated at two key residues, namely, Thr308 by 3-phosphoinositide-dependent kinase-1 (PDK1) (4) and Ser473 by the rictor-mammalian target of rapamycin (mTOR) complex (5). Activated Akt phosphorylates and regulates the function of many cellular proteins, including glycogen synthase kinase-3ß (GSK-3ß), Bcl-2 associated death domain (BAD), mTOR, and forkhead transcription factors. Through these signaling effectors, activation of PI3K and Akt results in diverse cellular processes including cell proliferation and survival, cell size/growth and response to nutrient availability, genome stability, tissue invasion and angiogenesis - processes that are critical for tumorigenesis (6). PTEN (also called MMAC1 or TEP1) is a 3-position lipid phosphatase that converts PIP_3 back to phosphatidylinositol-4,5-diphosphate (PIP_2) and thus shuts off PI3K/Akt signaling. Loss of PTEN function, due to somatic deletions or mutations of this tumor suppressor gene,

leads to an elevated concentration of the PIP_3 substrate, and consequent constitutive activation of Akt (6). In addition, somatic mutations of *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, have recently been shown to play an important role in the activation of the PI3K/Akt pathway (7,8). *PIK3CA* mutations have been frequently found in various human cancers, including colorectal, gastric, ovarian, breast, hepatocellular, and head and neck squamous cell carcinomas and glioblastomas (9-13), but rarely in lung cancer and melanoma (9,14,15). Most of these mutations are clustered in exon 9 (helical domain) and exon 20 (kinase domain) (9). Recently, a study on 40 Hong Kong NPC patients reported that no mutations were found in these 2 exons in all the NPC tumors (16). The frequency of *PIK3CA* mutations in NPC in Malaysian patients is unknown and its identification seems to be interesting because of the difference in geographical region, although the NPC incidence in Malaysia is one-third lower than that in Hong Kong.

Currently, the understanding of the mechanisms and consequences of Akt signaling in cancers is derived primarily from *in vitro* models using cancer cell lines and *in vivo* mouse models (6). In order to successfully translate these findings into molecular-targeted therapies, the assessment of Akt signaling pathway abnormalities in clinical samples is critical for the design of 'smart' clinical trials with molecularly targeted agents. Therefore, profiling of the Akt pathway *in vivo* is important in fully exploring its usefulness as a therapeutic target in NPC. To the best of our knowledge, only one published report has shown the activation of Akt and its target, GSK-3ß in 13 NPC specimens (17). In the present study, the role of the Akt signaling pathway and *PIK3CA* mutations in NPC tumorigenesis was determined by immunohistochemical and molecular techniques. The PTEN/PI3K/Akt/mTOR signaling pathway has been studied in breast cancer (18), but not in NPC. This is the first report suggesting that the increased activation of PI3K/Akt signaling in NPC is most likely due to overexpression of phosphorylated EGFR, rather than *PIK3CA* mutations since no mutations were detected in the NPC tissues. In addition, our data suggest that p-BAD and p-FKHR are correlated with p-Akt and there is no regulatory relationship of Akt with PTEN, GSK-3ß, and mTOR in NPC. Hence, our data suggest that the Akt signaling pathway in NPC pathogenesis is more complicated than the linear PTEN/ PI3K/Akt/mTOR pathway.

Materials and methods

Tissue specimens. Formalin-fixed, paraffin-embedded tissues from 64 NPC biopsy specimens and 38 nasopharyngeal mucosa biopsy specimens with no evidence of malignancy were collected from the Hospital of Kuala Lumpur, Malaysia. The NPC biopsy specimens collected between the years 2000 and 2004 were histologically classified into three types according to the World Health Organization classification: keratinizing squamous cell carcinoma (Type I; n=14), non-keratinizing carcinoma (Type II; n=23), and undifferentiated carcinoma (Type III; n=27).

Mutational analysis of PIK3CA. Among the collected biopsy specimens, 25 NPC and 2 non-malignant nasopharyngeal tissues were subjected to mutational analysis of *PIK3CA*. Genomic DNAs were isolated from the paraffin-embedded tissues using Qiagen® QIAamp DNA mini kit (Qiagen, Germany). The isolation procedures were done according to the manufacturer's instructions. Based on *PIK3CA* sequence data obtained from the NCBI GenBank (accession no. NM_006218), primers for exons 9 and 20 were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were synthesized by Invitrogen Corporation (Invitrogen, Hong Kong). The PCR amplification was performed using the following primers: exon 9 forward (F), 5'GCTAGAGACAATGAATTAAGGG 3'; exon 9 reverse (R), 5'GAGATCAGCCAAATTCAGTT3'; exon 20 (F), 5'TGGAATGCCAGAACTACAAT3'; exon 20 (R), 5'TTTGCCTGCTGAGAGTTATT3'. The amplified PCR products were subjected to direct sequencing by using the ABI PRISM® 3100 Genetic analyzer. All nucleotide changes were subsequently verified by bi-directional sequencing.

Immunohistochemistry. The paraffin-embedded tissues were serially sectioned to a thickness of 5 μm and the tissue sections were mounted on amino-silane coated slides. After heating at 60˚C for 1 h, the sections were deparaffinized in 2 times of xylene for 5 min each and rehydrated in graded ethanol (100, 95, 80 and 70%) for 3 min at each concentration. Then, the sections were washed in distilled water for 3 min. Tris-buffered saline with 0.1% Tween-20 (pH 7.6) was used in the following washing steps. Antigen unmasking was performed either by heat-induced epitope retrieval (HIER) or an enzyme digestion method depending on the antigens (Table I). After boiling with the appropriate buffer solution (10 mM citrate buffer pH 6.0, 1 mM EDTA buffer pH 8.0, or 10 mM Tris, 1 mM EDTA buffer pH 9.0; Table I) in a microwave oven for HIER, the sections were allowed to cool at room temperature for 25 min. For an enzyme digestion method, the sections were incubated in proteinase K. Endogenous peroxidase was blocked by 3% hydrogen peroxide. For sections pre-treated with the HIER method, the endogenous biotin was blocked by 0.01% avidin and followed by 0.01% D-biotin. After blocking with 3% bovine serum albumin for 1 h, the sections were incubated with a primary antibody for 1 h at room temperature or overnight (~18 h) at 4˚C. The primary antibodies and respective dilutions are listed in Table I. The immunoreactivity was detected by using the LSAB+ Kit (Dako, Carpinteria, CA) at room temperature according to the manufacturer's instructions. The procedures included 30-min incubation of biotinylated anti-rabbit, -mouse and -goat antibodies, followed by 30-min incubation of streptavidin-peroxidase. The 3,3'-diaminobenzidine (DAB) (liquid DAB+; Dako) solution was used as a chromogen. Sections were lightly counterstained with hematoxylin. Excluding the primary antibody to the sections served as the negative control. Positive/negative control tissues and cell pellets were used to optimize the staining protocol for the respective primary antibodies (Table I), in addition to act as the experimental control.

For detection of the p-EGFR antibody, a more sensitive biotinyl-tyramide-based signal amplification method was applied. The sections were incubated with the peroxidase-conjugated polymer carrying secondary antibodies (from

rimary antibodies used for immunohistochemical analysis.

Protein	Clone	Company	Catalog no.	Antigen retrieval	Positive/negative (+/-) control	Antibody dilution/ incubation time
EGFR	111.6	NeoMarkers (Fremont, CA)	MS-378-P1	Proteinase K (40 μg/ml), 12 min at 37˚C	Placenta (+) and HT-29 (+)	1:100/overnight
p-EGFR (Tyr1148)	Polyclonal	Cell signaling (Danvers, MA)	4404	EDTA buffer, 20 min	EGF-treated (+) and untreated (-) MDA-MB-468	1:50/overnight
PTEN	138G6	Cell signaling (Danvers, MA)	9559	Tris-EDTA, 20 min	PC-3 (-) and DU145 (+)	1:100/overnight
p-Akt (Ser473)	736E11	Cell signaling (Danvers, MA)	3787	Citrate buffer, 20 min	PC-3 (+), LNCaP (+), and LY294002-treated LNCaP (-)	1:50/overnight
p-Akt (Thr308)	244F9	Cell signaling (Danvers, MA)	4056	Citrate buffer, 20 min	LY294002-treated (-) and untreated (+) LNCaP	1:80/overnight
p-BAD (Ser136)	Polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-7999	Citrate buffer, 20 min	-	1:100/1 h
p-GSK-3ß (Ser9)	Polyclonal	Cell signaling (Danvers, MA)	9336	Citrate buffer, 20 min	-	1:100/overnight
p-FKHR (Ser256)	Polyclonal	Cell signaling (Danvers, MA)	9461	Citrate buffer, 20 min	LY294002-treated (-) and untreated (+) LNCaP	1:100/overnight
p-mTOR (Ser2448)	Polyclonal	Cell signaling (Danvers, MA)	2971	Citrate buffer, 20 min	-	1:100/overnight
Ki-67	MIB-1	Dako (Carpinteria, CA)	M7240	Tris-EDTA, 20 min	MCF-7 (+)	1:100/1 h

EnVision+ Dual Link Kit; Dako) for 30 min and after washing, they were incubated with biotinyl tyramide (from Tyramide Signal Amplification Biotin System, PerkinElmer Life and Analytical Sciences, MA) for 5 min. Then, the streptavidin-peroxidase and DAB were applied as mentioned above.

Preparation of sections from cell lines for validation of immunohistochemical staining. Cell lines, namely, HT-29, PC-3, DU145, and MCF-7 were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, sodium bicarbonate, penicillin and streptomycin. The cells with 70 to 90% confluence in a 75 cm^2 flask were trypsinized and spun down. The cell pellet was re-suspended in 1% melted agarose gel (37˚C). After the agarose gel solidified on ice, the agarose-embedded cell pellet was fixed with 10% neutral-buffered formalin, processed and embedded into a paraffin block. The sections of EGF-treated and untreated MDA-MB-468 cells and LY294002-treated and untreated LNCaP cells (kindly provided by Cell Signaling Technology, Inc.) were also used as controls in each of the staining experiments.

Immunohistochemical evaluation. A semi-quantitative scoring system adopted from previous studies (19) was used to evaluate the staining by all the antibodies. The degree of positive staining for all antibodies was evaluated by scoring on a scale of 0 to 4 for percentage of positive cells and on a scale of 0 to 3 for strength of staining intensity. The percentage of positive cells was evaluated using the following scale: 0, no staining of cells in any field; 1, positive staining in <25% of cells; 2, positive staining in 25-50%; 3, positive staining in 50-75%; and 4, positive staining in >75%. As for the evaluation of strength of staining intensity, it was evaluated using the following scale: 0, no staining of cells; 1$^+$, mild staining; 2$^+$, moderate staining and 3$^+$, strong staining. The final total score was generated by adding the score for the percentage of positive cells and the strength of staining intensity. Hence, the possible scores were 0 and 2-7. For immunostaining of PTEN and p-GSK-3ß, where the staining intensity was heterogeneous, a modified H-score was used (20). The percentage for each staining intensity in a section and staining intensity was scored accordingly. The H-score was calculated by the formula: H-score = (1 x Score for % mildly positive cells) + (2 x Score for % moderately positive cells) + (3 x Score for % strongly positive cells). The maximum possible score was 14. All tissues showed strong staining intensity (score 3) of PTEN in some stromal cells whereas moderate staining (score 2) in vascular endothelial cells, hence, these cells provided the internal positive control. Since the PTEN staining intensity of non-malignant

Table II. Immunohistochemical staining for the expression and phosphorylation of various biomolecules in NPC and non-malignant nasopharyngeal tissues.

	Number of immunoreactive cases (%)		Mean rank of score		
Biomolecules	NPC tissues (n=64)	Non-malignant nasopharyngeal tissues (n=38)	NPC tissues (n=64)	Non-malignant nasopharyngeal tissues (n=38)	p
EGFR[a]	37 (57.8)	13 (34.2)	57.20	41.89	0.009[d]
p-EGFR[a]	26 (40.6)	0 (0)	61.30	35.00	<0.001[d]
PTEN[b]	51 (79.7)	38 (100)	48.42	56.68	0.027[d]
p-Akt S[a]	23 (35.9)	0 (0)	61.59	34.50	<0.001[d]
p-Akt T[a]	27 (42.2)	0 (0)	60.12	36.99	<0.001[d]
p-FKHR[a]	24 (37.5)	18 (47.4)	48.59	56.41	0.175
p-BAD[a]	41 (64.1)	8 (21.1)	62.19	33.50	<0.001[d]
p-GSK-3ß[c]	17 (26.6)	4 (10.5)	56.30	43.42	0.029[d]
p-mTOR[a]	42 (65.6)	14 (36.8)	57.34	41.66	0.006[d]
Ki-67[a]	64 (100)	15 (39.5)	67.35	24.80	<0.001[d]

p-Akt S, p-Akt (Ser473); p-Akt T, p-Akt (Thr308). Statistical significance of the differences was analyzed using the Mann-Whitney's U-test. [a]Score ≥3, [b]Score ≥8 and [c]Score ≥6 were considered as positive staining. [d]P<0.05 indicates statistical significance.

nasopharyngeal cells was equal to that of the endothelial cells (score 2), the staining intensity of tumor cells was scored 1 if it was diminished relative to the endothelium, and scored 0 if it was undetectable while PTEN staining was present in the endothelial cells. The evaluation was performed independently by two experienced researchers, one of whom was a medically qualified histopathologist to ensure precision and accuracy.

Statistical analysis. Since the data were not normally distributed, analysis of the association of biomolecules with patient and tumor variables and the comparison of expression between NPC and non-malignant nasopharyngeal tissues were performed by the Mann-Whitney U-test or Kruskal-Wallis one-way analysis of variance by ranks. The Spearman rank correlation test was used to analyze the correlation among the expression of biomolecules. A 2-sided p-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 11.5 statistical software.

Results

Frequency of PIK3CA hotspot mutations. To determine whether *PIK3CA* is altered in NPC, mutational analysis of exons 9 and 20 of *PIK3CA* was done in 25 NPC and 2 non-malignant nasopharyngeal tissues. By direct DNA sequencing, 2 NPC tissues were found to have nucleotide changes at 1634 A → C (E545A) and 1658 G → C and a base deletion at nucleotide 1659T in exon 9 (Fig. 3B). Although these nucleotide alterations were not detected in the non-malignant nasopharyngeal tissues (Fig. 3A), they are most likely the artifacts generated by interferences from the sequence homologue at chromosome 22q11.2 and the Cat eye syndrome region, during PCR amplification. This event was reported by two previous studies (13,21). No mutations in exon 20 were detected in any of the NPC tissues. The two non-malignant nasopharyngeal tissues showed a wild-type sequence for both exons 9 and 20 of *PIK3CA*.

Aberrant expression and phosphorylation of PI3K/Akt signaling pathway-related biomolecules. In order to elucidate the potential involvement of Akt signaling in NPC, we examined by immunohistochemistry the related biomolecules in the signaling pathway in 64 NPC and 38 non-malignant nasopharyngeal tissues. In the NPC tissues, the expression of EGFR (57.8%), p-EGFR (40.6%), p-Akt (Ser473) (35.9%), p-Akt (Thr308) (42.2%), p-BAD (64.1%), p-GSK-3ß (26.6%), p-mTOR (65.6%) and Ki-67 (100%) was significantly higher compared to the non-malignant nasopharyngeal tissues (Table II; Fig. 1B and C). However, there was no statistically significant difference in p-FKHR (37.5% of NPCs; P=0.175) expression between NPC and non-malignant nasopharyngeal tissues (Table II; Fig. 1C). The NPC tissues which are positive for p-EGFR showed EGFR expression with at least a score of 2. To define the frequency of PTEN loss in NPC tissues, the score was compared with the non-malignant nasopharyngeal tissues. Since 37 out of 38 and 1 out of 38 of the non-malignant nasopharyngeal tissues have a score of 8 and 6, respectively, for the PTEN expression in the epithelium, a score of ≥8 in tumor tissues was considered as a normal expression (Fig. 1Aa), a score of <8 as reduced expression, and a score of 0 as loss of PTEN expression. All the NPC and non-malignant nasopharyngeal tissue showed uniform PTEN staining in stromal cells, which acted as an internal control to ascertain the quality of antigen preservation. A complete loss of PTEN staining was observed in 4.7% (3 cases) of tumors (Fig. 1Ac), whereas reduced expression was seen in 15.6% (10 cases) (Fig. 1Ab). The PTEN scoring in NPC tissues was significantly lower than that in non-malignant nasopharyngeal tissues (P=0.027) (Table II).

The specificity of PTEN and the phosphorylation-specific antibodies was validated in a defined *in vitro* cell line system. Due to an insufficient amount of paraffin sections of the cell line samples, only the phospho-specific antibodies against p-EGFR, p-Akt (Ser473), p-Akt (Thr308), and p-FKHR were validated. PC-3 cells, which have a homozygous deletion of

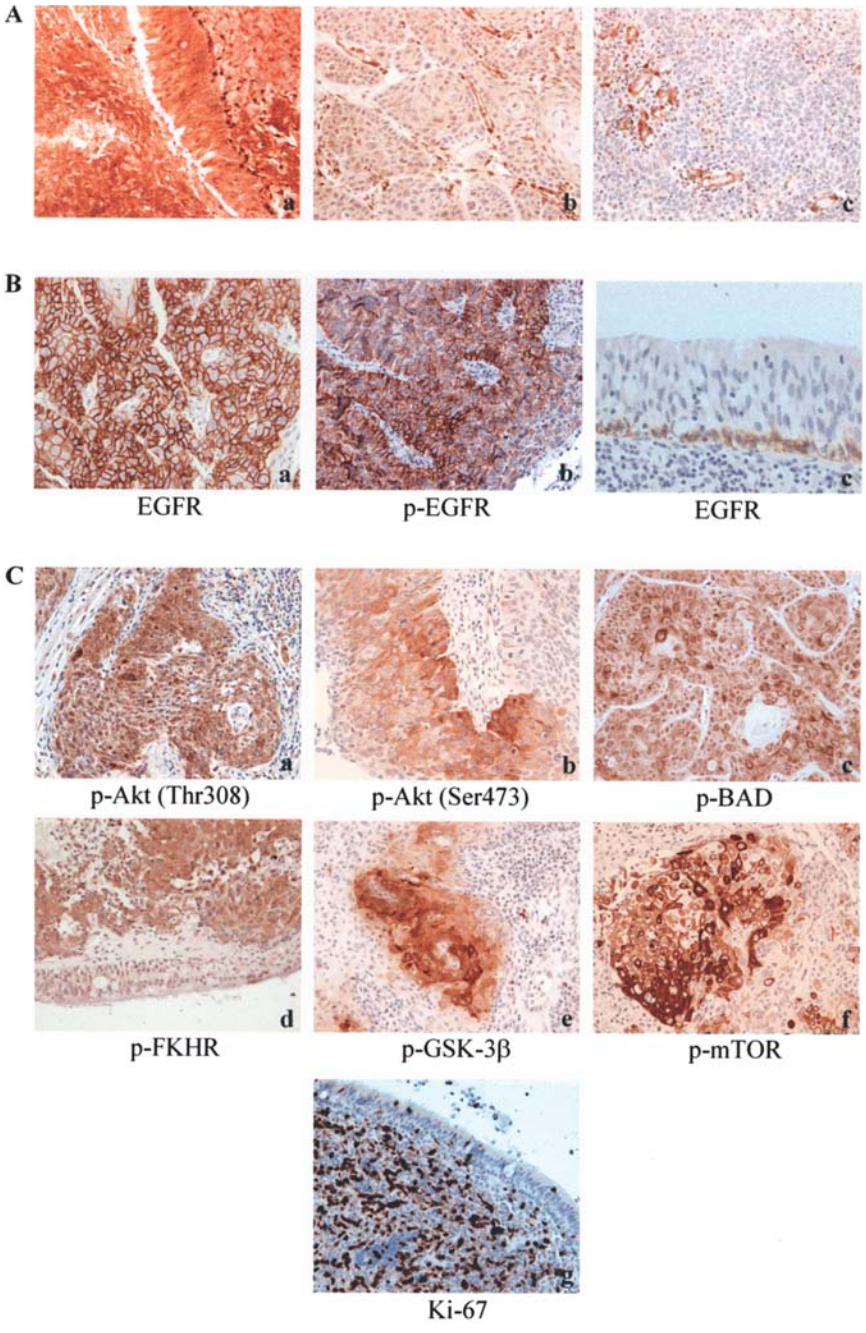

Figure 1. Immunohistochemical staining of the Akt pathway-related biomolecules. (A) PTEN protein expression levels observed in NPC by immunohistochemistry. (a) Normal staining intensity (score 2). (b) Tumor cells show equal staining intensity to normal adjacent epithelium. (c) Some stromal cells demonstrating intense staining (score 3) (x200 magnification). (B) Reduced staining intensity (score 1). Tumor cells showing staining intensity less than that observed in the stromal cells (x200). (c) Loss of staining. No staining is seen in tumor cells (x200). Expression of EGFR and p-EGFR was observed in tumors (a-b, x200). The basal cells of normal nasopharyngeal mucosa were mildly stained for EGFR (c, x400). (C) The phosphorylation of Akt on Thr308 and Ser473 and its downstream molecules including BAD, FKHR, GSK-3ß, and mTOR were also detected by immunohistochemistry in tumors (a-f, x200). The apparently normal adjacent epithelium showed an absence of p-Akt (Ser473) and mild staining of p-FKHR, in contrast to a higher level of both molecules in the tumor area. Most tumor cells showed higher proliferative activity than the adjacent normal epithelial cells, as indicated by intense Ki-67 staining (g, x200).

PTEN was not stained while DU145 cells, which contain a wild-type PTEN allele, were positively stained (Fig. 2A). The EGF-treated and untreated MDA-MB-468 cells, which act as a positive and negative control for p-EGFR staining, respectively, showed an expected result (Fig. 2C). The immunostaining of p-Akt (Ser473) and p-Akt (Thr308) was reduced in LY294002-treated LNCaP cells compared to the untreated cells (Fig. 2B). We noted that the staining intensity of p-FKHR (Ser256) was not affected in LY294002-treated LNCaP cells (not shown).

Correlations between EGFR, p-EGFR, PTEN and p-Akt. Perturbations of the Akt pathway, as indicated by an increased level of phosphorylated Akt, was detected in our NPC tissues. To dissect the molecular events that are responsible for Akt activation in NPC, the protein expression of EGFR, p-EGFR, PTEN and p-Akt was correlated statistically using the Spearman rank correlation test (Table III). The p-EGFR correlated with an increased level of p-Akt (Thr308) (P=0.005) but not with p-Akt (Ser473) (P=0.393). However, no correlation was observed between EGFR and p-Akt

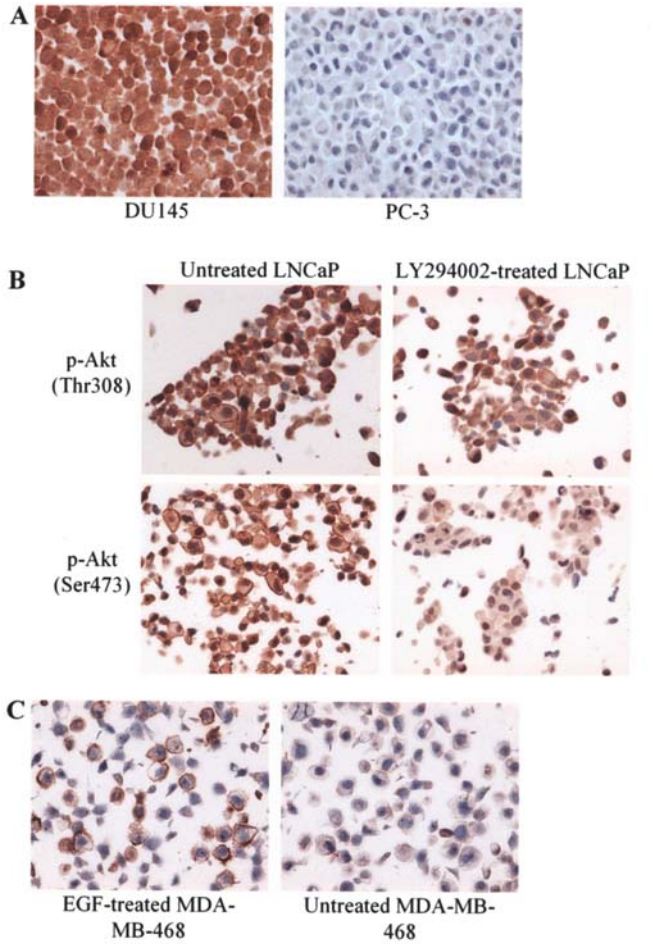

Figure 2. Validation of PTEN and the phospho-specific antibodies. (A) The DU145 cells were specifically stained by the PTEN antibody, as no staining was shown in PC-3 cells (x400). (B) Both phosphorylation sites of Akt (Ser473 and Thr308) were detected in LNCaP cells and their staining was reduced in LY294002-treated LNCaP cells (x400). (C) The p-EGFR staining was induced in EGF-treated MDA-MB-468 cells, characterizing the specificity of the antibody (x400).

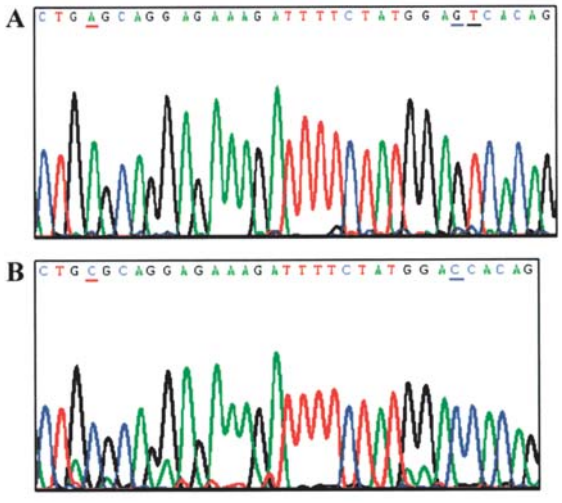

Figure 3. Sequence analysis of the *PIK3CA* exon 9. (A) The wild-type sequence was found in a non-malignant nasopharyngeal tissue. (B) Identification of the A1634C (red underline) and G1658C transitions (blue underline) and a deletion of nucleotide 1659T (black underline) in NPC tissue. These nucleotide changes were most probably generated from the sequence of putative PIK3CA pseudogene at chromosome 22q11.2, the Cat eye syndrome region, during PCR amplification.

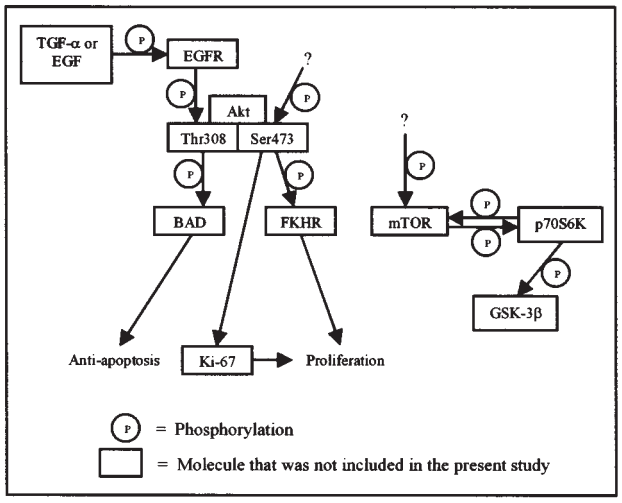

Figure 4. The potential interactions between the biomolecules in the PI3K/Akt signaling pathway as proposed from this study and previous reports. The activation of EGFR leads to the phosphorylation of Akt at Thr308 but not at Ser473. Phosphorylation of Akt at Thr308 correlates with p-BAD resulting in anti-apoptosis. Ser473 phosphorylation of Akt is induced by an unknown mechanism. A positive correlation of p-Akt (Ser473) with FKHR phosphorylation and Ki-67 expression results in cell survival and proliferation. P-GSK-3ß and p-mTOR are not correlated with Akt phosphorylation. A correlation was found between p-GSK-3ß and p-mTOR. Since the activated mTOR phosphorylates p70S6K (43), it is possible that p-p70S6K may phosphorylate GSK-3ß as supported by Zhang *et al* (44).

(Ser473) or p-Akt (Thr308) immunoreactivity. It is noteworthy that PTEN loss was correlated with overexpression of EGFR (P=0.014), but not with p-Akt (Ser473) or p-Akt (Thr308) level.

Correlation of p-Akt with its downstream molecules. We then examined the relationship between the Akt activation and its downstream targets: p-BAD, p-FKHR, p-GSK-3ß and p-mTOR, and its biological effect on tumor cell proliferation as indicated by Ki-67. Using the Spearman rank correlation test, a statistically significant positive correlation between p-Akt (Thr308) and p-BAD level (P=0.004) was identified (Table III). P-FKHR and Ki-67 both showed a positive correlation with p-Akt (Ser473) immunoreactivity (P=0.001 and P=0.004, respectively). No statistically significant correlation was seen between either p-Akt (Thr308) or p-Akt (Ser473) and p-GSK-3ß or p-mTOR level.

Since phosphorylation of Akt on both Ser473 and Thr308 has been shown to be required for full activation of the kinase (5,22), we categorized the NPC cases into 4 groups, one group is positive for both Ser473 and Thr308, and the other groups are for Ser473 only, Thr308 only and none of them, respectively. We then statistically tested whether the four groups of Akt phosphorylation differ in the scoring of downstream molecules by using the Kruskal-Wallis test. The group with phosphorylated Akt on both Ser473 and Thr308 had significantly higher level of p-BAD and p-FKHR immunoreactivity (P=0.039 and P=0.013, respectively), compared to the group with either Ser473 alone or Thr308 alone and with non-phosphorylated Akt (Table IV).

The analysis of the relationship among p-Akt, its upstream and downstream molecules also demonstrated the positive

Correlation among the total score of biomolecules in NPC.

		p-EGFR score	PTEN score	p-Akt S score	p-Akt T score	p-FKHR score	p-BAD score	p-GSK-3ß score	p-mTOR score	Ki-67 score
EGFR	C.C	0.455	-0.307	-0.238	-0.060	-0.297	-0.178	-0.171	-0.117	-0.188
score	p	< 0.001[a]	0.014[a]	0.058	0.635	0.017[a]	0.160	0.177	0.356	0.136
p-EGFR	C.C		-0.144	0.109	0.349	-0.107	0.118	-0.064	0.175	-0.160
score	p		0.255	0.393	0.005[a]	0.400	0.352	0.616	0.168	0.206
PTEN	C.C			0.183	-0.020	0.211	0.230	0.160	-0.152	0.185
score	p			0.149	0.877	0.094	0.068	0.208	0.230	0.144
p-Akt S	C.C				0.379	0.408	0.132	0.055	-0.132	0.351
score	p				0.002[a]	0.001[a]	0.299	0.664	0.300	0.004[a]
p-Akt T	C.C					0.151	0.354	-0.154	0.094	0.052
score	p					0.235	0.004[a]	0.223	0.462	0.684
p-FKHR	C.C						0.149	0.068	-0.021	0.090
score	p						0.241	0.591	0.867	0.478
p-BAD	C.C							-0.028	0.146	0.292
score	p							0.828	0.249	0.019[a]
p-GSK-3ß	C.C								0.294	0.118
score	p								0.018[a]	0.353
p-mTOR	C.C									-0.186
score	p									0.142

Spearman rank correlation test. [a]A 2-sided p<0.05 indicates a statistical significance. p-Akt S, p-Akt (Ser473); p-Akt T, p-Akt (Thr308); C.C , Correlation coefficient.

Table IV. Association between Akt phosphorylation and the total score of downstream targets in NPC.

		Akt phosphorylation				
		S473 and T308 (n=14)	S473 only (n=9)	T308 only (n=13)	None (n=28)	p
p-mTOR	M.R	29.96	29.39	41.31	30.68	0.256
p-BAD	M.R	42.21	25.17	37.38	27.73	0.039[a]
p-GSK-3ß	M.R	31.64	38.78	30.35	31.91	0.733
p-FKHR	M.R	44.04	37.89	26.62	27.73	0.013[a]

Statistical significance of the differences was analyzed using the Kruskal-Wallis test. [a]P<0.05 indicates a statistical significance. M.R, mean rank of score.

correlation of p-BAD with Ki-67 (P=0.019) and p-mTOR with p-GSK-3ß (P=0.018) and the negative correlation of EGFR with p-FKHR (P=0.017) (Table III).

Association of Akt pathway-related biomolecules with patient and tumor variables. The immunostaining of the biomolecules in the Akt pathway was associated with patient and tumor variables including age at diagnosis, gender, race and histological type. The level of p-Akt (Thr308) in NPC samples was significantly associated with female patients (p<0.001). No significant association of other biomolecules with the patient and tumor variables was found (data not shown).

Discussion

We have successfully demonstrated that the Akt pathway can be examined *ex vivo* with the immunohistochemical technique by using formalin-fixed, paraffin-embedded NPC tissues. Our data show that the frequency of PTEN loss (4.7%) is ten times lower compared to a previous study (23). No correlation between PTEN loss and Akt activation was found in our study, which is consistent with a report on breast cancer (24). This is in contrast to earlier studies on prostate, breast and endometrial cancers and glioblastoma, showing increased p-Akt in tissues with PTEN loss (25-28). It is worth noting that the finding of a positive correlation between the p-Akt level and PTEN expression has also been reported in breast and ovarian cancers (18,29). These inconsistent findings might be due to various types of antibodies used against PTEN in immunohistochemical assay. Indeed, Pallares *et al* (30) reported that different PTEN antibodies show a difference in specificity and ability to correlate with its molecular abnormalities and phosphorylated Akt. Instead of PTEN loss, loss of promyelocytic leukemia (PML) protein may also lead to the increase of p-Akt level and function *in vitro* and *in vivo* (31). Loss of PTEN staining might reflect PTEN inactivation due to various mechanisms including homozygous deletions, nonsense mutations, certain internal deletions, promoter

methylation, lack of transcription factor, and post-transcriptional modifications. Notably, by using antibody-based analysis, the true frequency of PTEN inactivation may be underestimated, since genetic alterations such as missense mutations in PTEN may produce a functional compromised molecule without any alteration in protein expression (32). We could not determine whether mutations exist as we did not have sufficient biopsy material.

Functional analysis has shown that the hotspot mutations in *PIK3CA* enhance the lipid kinase activity *in vitro* as compared with wild-type and an increase of Akt phosphorylation in colorectal cancer cell lines (7,8). Consistent with these reports, the frequency of *PIK3CA* mutations was significantly higher in p-Akt-positive than in p-Akt-negative human breast cancer tissues (33). To examine whether the Akt phosphorylation resulted from *PIK3CA* mutations in the present study, 25 NPC and 2 non-malignant nasopharyngeal tissues were analyzed for the sequence changes of exons 9 and 20 of *PIK3CA*. In concordance with a previous report (16), no mutations in exon 9 or 20 were detected in any of the tested NPC tissues. Therefore, the activation of Akt in NPC may be due to other mechanisms, such as growth factor activation, rather than *PIK3CA* hotspot mutations.

The EGFR is a membrane-bound tyrosine kinase and frequently overexpressed in various types of cancer, including NPC, as shown in the present study (Table II). The negative correlation of EGFR with PTEN indicates that their expression occurs frequently in cases with a reduction/absence of PTEN. Loss of function of PTEN could contribute to the development of resistance to receptor tyrosine kinase inhibitors (e.g. gefitinib) (34); therefore, targeting these receptors might not be a good design of molecular-targeted therapy for most of the patients with PTEN-downregulated NPC. EGFR exerts effects by the activation of a complex series of adapter molecules and kinases after exposure to various ligands. For instance, EGF-induced activation of EGFR leads to the phosphorylation of the other ErbB family members resulting in recruitment and activation of the p85 subunit of PI3K (3). Our present study shows that the p-EGFR is positively correlated with p-Akt (Thr308), but not with Ser473 phosphorylation. This observation is supported by a published study, in which Thr308 is the phosphorylation site responsible for the activation of Akt by PI3K-generated lipid product, PIP_3 and the upstream kinase, PDK1 (35); however, this may be controversial as other reports suggest that PIP_3-dependent translocation of Akt to the plasma membrane promotes Ser473 phosphorylation, which is, in turn, necessary for PDK1-mediated phosphorylation of Thr308 (5,22).

Since phosphorylation of Akt on both Ser473 and Thr308 has been shown to be required for full activation of the kinase (5,22), we evaluated the level of p-Akt (Ser473) and p-Akt (Thr308) to determine the activation status of Akt. We also examined the correlation when both Ser473 and Thr308 was phosphorylated or when only one of these residues was phosphorylated with the downstream molecules. Although we observed that p-Akt (Ser473) correlates with p-FKHR, whereas p-Akt (Thr308) is correlated with p-BAD (Table III), the level of both Akt substrates is significantly higher in tumors that co-express p-AktSer473 and p-AktThr308 than those that express one of them (Table IV). These findings suggest a mechanism in which the activated EGFR could lead to the activation of Akt through PI3K, and subsequently to phosphorylation of BAD. This protein, in its dephosphorylated form, interacts with a Bcl family member (Bcl-2 or Bcl-xL) and induces apoptosis (36); however, after BAD is phosphorylated at Ser136 by p-Akt, it associates with 14-3-3 proteins, thus preventing p-BAD from interacting with Bcl-2 or Bcl-xL and subsequent apoptosis (36). Thus, BAD inactivation promotes cell survival. The correlation between phosphorylated BAD and Ki-67 in the present study supports the notion that the tumor cells proliferate inappropriately without compensatory apoptosis. The disruption of normal cell function and tissue homeostasis that maintains the balance between cellular proliferation and apoptosis is a feature of tumor development and progression.

In our NPC tissues, we observed a positive correlation between p-Akt and p-FKHR (Ser256), which is consistent with previous reports that showed similar findings in non-small-cell lung carcinoma and glioblastoma (26,37). Ser256 phosphorylation of this forkhead transcription factor by Akt may result in the nuclear exclusion and impairment of DNA binding activity (38), thus inhibiting the expression of FOXO-regulated genes, which control the cell cycle, cell death, cell metabolism and oxidative stress. Unexpectedly, the non-malignant tissues have an equally high level of p-FKHR (Ser256) to those of NPC tissues even though, no immunoreactivity of p-Akt (Ser473) or p-Akt (Thr308) was detected. Although the FKHR-Ser256 is a well-known target of p-Akt, we cannot entirely exclude the possibility that another kinase might closely regulate FKHR phosphorylation in normal nasopharyngeal tissue, which is not known at this time. On the other hand, this raises the issue of specificity of the antibody used. We noted that the LNCaP cells were positively stained with p-FKHR (Ser256), but no significant reduction of immunostaining in LY294002-treated LNCaP cells was seen (not shown). This observation differs from a previous report showing that the low intensity of p-FKHR in LNCaP cells was completely abolished by the LY294002 treatment (28). This difference could probably be due to different phosphorylation sites of p-FKHR detected in the study (Thr24 of FKHR or Thr32 of FKHRL1), which might be more dependent on PI3K-mediated phosphorylation compared to Ser256. Nevertheless, the effect of LY294002 on p-FKHR (Ser256) level in LNCaP cell requires further confirmation by using Western blot analysis.

Our result demonstrating the correlation of p-Akt (Ser473) with the proliferation marker, Ki-67 supports the oncogenic role of the activated Akt in promoting cell cycle progression *in vivo*. This cellular process may be a result from the inactivation of FKHR, since p-FKHR correlates with p-Akt (Ser473). However, p-FKHR was found not to be correlated with Ki-67, suggesting that FKHR inactivation alone is unable to account for cell proliferation. This suggests that other downstream molecules of Akt may exert this effect. Akt activation also mediates cell cycle progression by phosphorylation and consequent inhibition of GSK-3ß to avert cyclin D1 degradation (39). Moreover, phosphorylation of Akt/mTOR kinases also results in an increased translation of cyclin D1, D3, and E transcripts (40). However, we show that neither p-Akt (Ser473) nor p-Akt (Thr308) correlates with the

3 (Ser9) and p-mTOR (Ser2448). This indicates that ated proliferation is probably independent of its downstream substrate p-GSK-3ß (Ser9) or p-mTOR (Ser2448) in NPC, suggesting that other possible pathways may be involved such as through the phosphorylation of the cell cycle inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ (41). Phosphorylation of mTOR by Akt still remains elusive. In addition to the direct phosphorylation of mTOR by the activated Akt, perturbation of the TSC/Rheb pathway may influence mTOR Ser2448 phosphorylation (42). Furthermore, Chiang and Abraham (43) have shown that mTOR Ser2448 is directly phosphorylated by p70 ribosomal S6 kinase (p70S6K) *in vitro* and this phosphorylation event is not obligatorily linked to Akt. This Ser2448 phosphorylation reflects a feedback signal to mTOR from its downstream target, p70S6K. As mTOR activity is essential for the activation of p70S6K, p-mTOR (Ser2448) is therefore an indicator of the level of mTOR signaling.

A published report has shown that activated Akt and p-GSK-3ß are highly expressed in NPC (17). However, the correlation between these two molecules was not reported in that study. For the first time, we investigated the correlation of activated Akt with p-GSK-3ß but found no correlation in our NPC tissues. Therefore, this raises the possibility that other protein kinases rather than Akt would be responsible for the phosphorylating and inactivating GSK-3ß in NPC. We found a correlation between p-GSK-3ß (Ser9) and p-mTOR (Ser2448). Since p-mTOR (Ser2448) is the indicator of the p70S6K kinase activity (43), this finding may suggest a possible role for p70S6K which is a downstream substrate of mTOR in phosphorylation of GSK-3ß. Recently, the p70S6K, rather than Akt, was found to phosphorylate and inactivate GSK-3ß in mTOR-active cells (44). This study further supports our hypothesis that p70S6K is the GSK-3ß regulatory kinase in NPC.

Our finding of an association between p-Akt (Thr308) and female patients is consistent with the published study on non-small cell lung cancer (45). Furthermore, Saraiva *et al* (46) reported that the absence of p-Akt expression was correlated with male gender in uveal melanoma, which may indirectly support our findings. The implications of these observations are not known and need to be further investigated.

The correlation analysis has provided an important hint on the interaction of signaling molecules *in situ* on tumor tissues (Fig. 4). In conclusion, our study provides evidence suggesting that the activation of the EGFR/PI3K/Akt pathway may be involved in NPC pathogenesis. In addition, PTEN loss is not associated with Akt activation and the activated Akt seems unlikely to play a role in the phosphorylation of mTOR and GSK-3ß in NPC. Hence, the simple model of linear PTEN/PI3K/Akt/mTOR pathway is unlikely to be involved in NPC tumorigenesis. However, the interactive network of PTEN loss, mTOR activation, and GSK-3ß inhibition in NPC is unclear and needs to be further elucidated. Our data imply that only a portion of NPC patients (35-40%), particularly those with no functional loss of PTEN, would be suitable for treatment with inhibitors of EGFR, Akt, and PI3K if these biomarkers, namely, p-EGFR, p-Akt and PI3K are used as a guide to predict drug responses. Further investigations will be required to support this statement.

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