Pathol. Oncol. Res. (2016) 22:413–419
DOI 10.1007/s12253-015-0007-8

ORIGINAL ARTICLE

Increased Expression of Phosphatidylinositol 3-Kinase p110α and Gene Amplification of *PIK3CA* in Nasopharyngeal Carcinoma

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Received: 12 March 2015 / Accepted: 4 November 2015 / Published online: 18 November 2015

Abstract Molecular alterations in *PIK3CA* oncogene that encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K p110α) are commonly found in human cancers. In this study, we examined the expression of PI3K p110α and *PIK3CA* gene amplification in 74 nasopharyngeal carcinoma (NPC) cases. Immunohistochemical staining demonstrated overexpression of PI3K p110α protein in 44.6 % (33/74) of NPCs and 4.8 % (2/42) of the adjacent normal nasopharyngeal mucosa. Copy number of *PIK3CA* gene was successfully analyzed in 51 of the total NPC cases and 19 non-malignant nasopharynx tissues by quantitative real-time PCR. Using mean + 2(standard deviation) of copy numbers in the non-malignant nasopharynx tissues as a cutoff value, *PIK3CA* copy number gain was found in 10 of 51 (19.6 %) NPC cases. High PI3K p110α expression level was correlated with increased *PIK3CA* copy number (Spearman's rho =0.324, $P = 0.02$). PI3K p110α expression and *PIK3CA* copy number did not associate with Akt phosphorylation, and patient and tumor variables. This study suggests that PI3K p110α overexpression, which is attributed, at least in part, to *PIK3CA* gene amplification, may contribute to NPC pathogenesis. However, these molecular aberrations may not be responsible for activation of Akt signaling in NPC.

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Keywords *PIK3CA* · Akt · Nasopharyngeal carcinoma · Immunohistochemistry

Introduction

Aberrant activation of phosphatidylinositol 3-kinase (PI3K) pathway is one of the most common molecular alterations in human malignancy. PI3K is activated in cells exposed to various stimuli such as cytokines, hormones, growth factors, and extracellular matrix components. PI3K exerts its oncogenic role by activating diverse signaling cascades that regulate cell proliferation and survival, cell response to nutrient availability, glucose metabolism, cell migration and invasion, genome stability, and angiogenesis [1].

PI3K is a heterodimer comprising of a p85 (85 kDa) regulatory subunit and a p110 (110 kDa) catalytic subunit. Upon ligand-mediated activation of receptor tyrosine kinase (RTK), inactive p85-p110 complex in the cytoplasm is recruited to the membrane via p85–RTK interaction. At the cell membrane, p110 subunit catalyzes the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3), which is essential for the activation of several downstream effectors, including Akt (also known as protein kinase B) and serum and glucocorticoid-regulated kinase (SGK) families of kinases [2, 3]. There are four p110 isoforms have been characterized, namely, p110α, p110β, p110δ, and p110γ, which are distinctly encoded by *PIK3CA*, *PIK3CB*, *PIK3CD*, and *PIK3CG* genes, respectively.

PIK3CA activating mutations, mostly clustered in exons 9 (helical domain) and 20 (kinase domain), have been frequently found in various human cancers, including breast, colorectal, endometrial, head and neck, ovarian, and gastric cancers and glioblastomas, but rarely in lung cancer and melanoma [4]. The *PIK3CA* mutations are shown to confer oncogenic

transforming activity and promote cancer cell growth and invasion, suggesting that *PIK3CA* is a promising therapeutic target [5, 6].

An alternative genetic alteration to mutation in *PIK3CA* is the gene amplification or gain of gene copy number, which was also found in human cancers, such as head and neck, cervical, ovarian, breast, gastric, esophageal, and lung carcinomas [7–9]. *PIK3CA* amplification might result in overexpression of its gene product, p110α catalytic subunit, leading to the activation of downstream pathways of PI3K [10].

Nasopharyngeal carcinoma (NPC) is a rare head and neck cancer in the United States and Western Europe, but is common in Southern China and parts of Southeast Asia [11]. Our previous studies revealed that the epidermal growth factor receptor (EGFR)-activated Akt signaling pathway may involve in NPC pathogenesis [12] and induce NPC cell invasion via PI3K [13]. Our results also indicate that the activation of Akt in NPC may not be due to *PIK3CA* mutation as no hotspot mutations were found in any of the samples tested [12]. Other reported frequencies of *PIK3CA* mutations in exons 9 and 20 vary from no evidence of mutations to 9.6 % of NPC cases [14–16]. To date, only one published study, which was conducted in Tunisia, has reported the *PIK3CA* amplification in NPC [17] and the overexpression of PI3K p110α has not yet been reported in NPC. In the present study, we determined the frequency of PI3K p110α overexpression and *PIK3CA* amplification and their relationship in NPC. The associations of these molecular alterations with Akt phosphorylation and patient and tumor variables were also investigated. This study may provide further understanding of the molecular pathogenesis of NPC that will assist in identifying relevant therapeutic targets.

Materials and Methods

Tissue Specimens and Blood Samples

Formalin-fixed, paraffin-embedded (FFPE) tissues from 74 NPC biopsy specimens and 19 nasopharyngeal mucosa biopsy specimens with no evidence of malignancy were collected from the Kuala Lumpur Hospital, Malaysia, between the years 2000 and 2004. Ten individual blood samples were collected from normal donors for the *PIK3CA* copy number analysis. The sample collection for this study was approved by our ethics committee.

Immunohistochemistry

Four-μm-thick FFPE tissue sections from the 74 NPC specimens were deparaffinized and then boiled with 10 mM Tris, 1 mM ethylenediamine tetraacetic acid (EDTA) buffer (pH 9.0) in a microwave oven for 20 min. After blocking the endogenous peroxidase with 3 % hydrogen peroxide, the sections were incubated with 3 % bovine serum albumin (BSA) for 1 h. Primary anti-PI3K p110α antibody (1:400 dilution, clone C73F8; Cell Signaling Technology, Danvers, MA) was applied to each section and incubated for 2 h at room temperature. The immunoreactivity was detected by using the Lab Vision™ UltraVision™ LP Detection System: HRP Polymer and then the 3,3′-diaminobenzidine (DAB) solution (Lab Vision™ DAB Plus Substrate Staining System) as a chromogen, according to the manufacturer's instructions (Thermo Fisher Scientific, Fremont, CA). Sections were counterstained lightly with hematoxylin. Negative control was prepared by excluding the primary antibody.

According to a published study with minor modifications [18], the PI3K p110α immunostaining on each entire slide was evaluated semi-quantitatively under a light microscope using the following scores: 0, no staining at all or weak staining of ≤25 % of cells; 1+, weak staining of >25 % of cells or moderate staining of ≤50 % of cells; 2+, moderate staining of >50 % of cells or strong staining of ≤50 % of cells; and 3+, strong staining of >50 % of cells. The endothelial cells with strong PI3K p110α staining in the tissues were used as an internal positive control and a reference for determining the degree of staining intensity. The specificity of the anti-PI3K p110α antibody had been validated as reported in our previous study [19].

PIK3CA Copy Number Analysis by Quantitative Real-Time PCR

Genomic DNAs were extracted from the 5-μm-thick FFPE tissue sections and the whole bloods using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The primer and TaqMan probe sequences (5′ to 3′) adopted from a previous study [20] were as follows: *PIK3CA* (forward) AAATGAAGCTCACTCTGGATTCC, (reverse) TGTGCAATTCCTATGCAATCG, and (TaqMan) 6-carboxyfluorescein-CACTGCACTGTTAATAACTCTC AGCAGGCAAA-tetramethylrhodamine; housekeeping gene *ACTB* (forward) TCACCCACACTGTGCCCATCTACGA, (reverse) TCGGTGAGGATCTTCATGAGGTA, and (TaqMan) 6-carboxyfluorescein-ATGCCCTCCCC CATGCCATCC-tetramethylrhodamine. All the primers and TaqMan probes were purchased from AITbiotech (Singapore). The real-time PCR in 25-μl reaction mixture containing 1× Taq buffer with KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer (forward and reverse), 240 nM TaqMan probe, 0.05–6 ng genomic DNA, and 1.25 U Taq DNA polymerase (Fermentas; Thermo Fisher Scientific, Glen Burnie, MD) was performed in duplicate for each sample on the Mastercycler ep *realplex*^{4} (Eppendorf, Hamburg, Germany). The thermal cycling consisted of a 5-min initial denaturation at 95 °C, followed by 40 cycles of denaturation

(95 °C for 20 s) and simultaneous annealing and extension (60 °C for 1 min). Standard curves were generated for *PIK3CA* and the reference gene *ACTB* using 5-fold serial dilutions of pooled whole blood DNA (from 3 normal donors). The amount of *PIK3CA* and *ACTB* in the samples was calculated using their C_T values and the corresponding standard curve. The amount of *PIK3CA* was divided by the amount of *ACTB* to obtain the normalized *PIK3CA* amount. The relative *PIK3CA* copy number was calculated by dividing the normalized *PIK3CA* amount by the value of the calibrator, which was a whole blood DNA sample (same sample included in every assay). The relative copy number was then multiplied by 2 to consider that 2 is the copy number of normal diploid DNA. T-47D breast cancer cell line harboring *PIK3CA* amplification (~5 copies) was included in every assay as a positive control and to monitor the assay consistency.

Statistical Analysis

Mann–Whitney U-test was performed to assess the difference in PI3K p110α expression between NPC and normal adjacent mucosal tissues. Unpaired t test with Welch's correction was used on the log-transformed *PIK3CA* copy number to assess the difference in copy number between NPC and non-malignant nasopharynx tissues. Analysis of the correlations among *PIK3CA* copy number, PI3K p110α expression, and Akt phosphorylation in NPC tissues was performed by Spearman's rank correlation test. The associations of *PIK3CA* copy number and PI3K p110α expression with patient and tumor variables were assessed by Fisher's exact test, Freeman–Halton extension of the Fisher's exact test, Mann–Whitney U-test or Kruskal–Wallis test. SPSS version 11.5 statistical software (SPSS Inc., Chicago, IL) was used to conduct

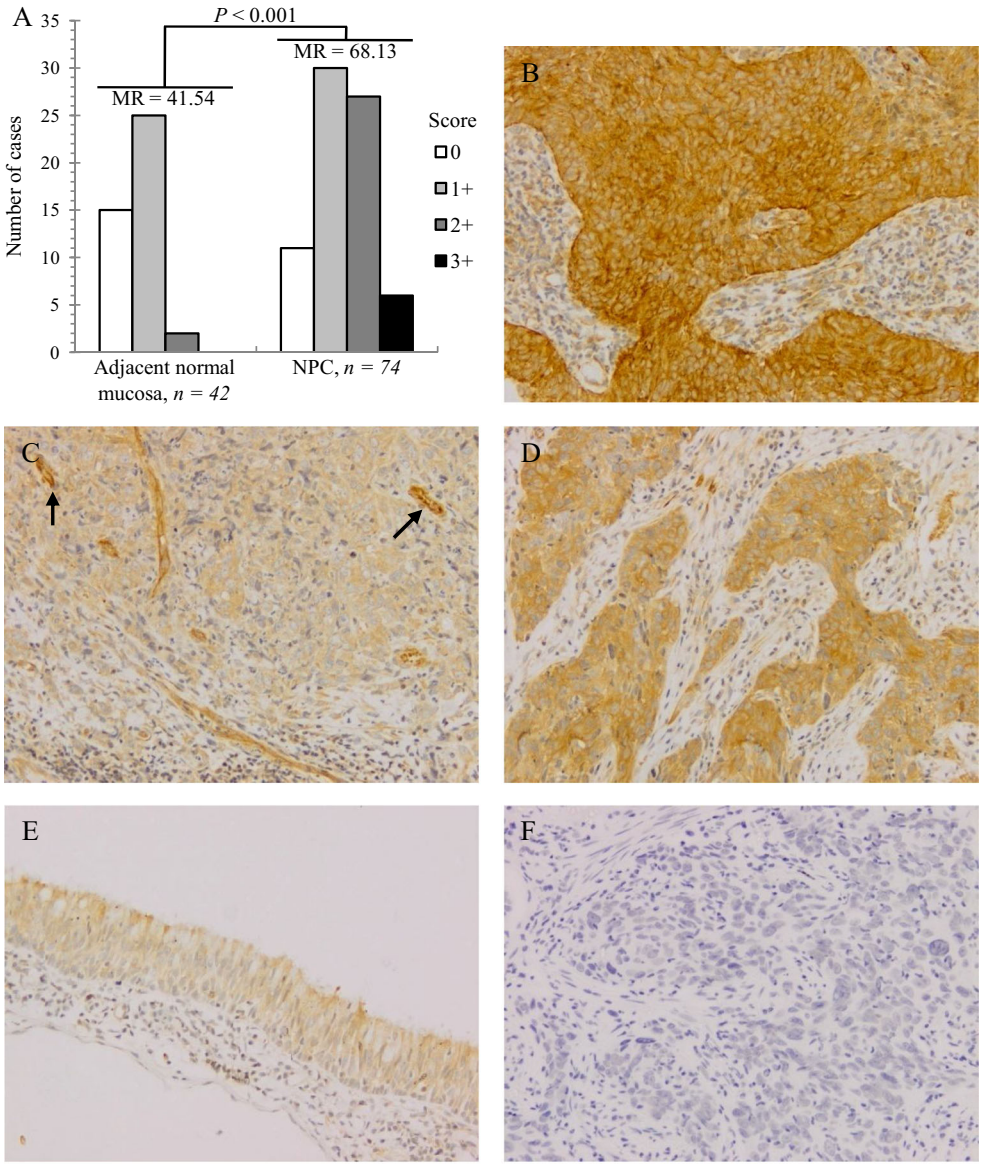

Fig. 1 Immunohistochemical staining of PI3K p110α in NPC and histologically normal adjacent nasopharyngeal mucosa. **a** Mann-Whitney U-test was used to compare the scoring values of PI3K p110α immunoreactivity between NPC and normal adjacent mucosal tissues. MR, mean rank score. Representative areas showing **b** strong, **c** mild, and **d** moderate cytoplasmic and membranous immunostaining of PI3K p110α in tumor tissues. Strong PI3K p110α immunostaining was also observed in adjacent endothelial cells (*arrow*). **e** A normal adjacent mucosal tissue shows mild staining. **f** A negative control demonstrates no immunoreactivity. Original magnification ×200 (**b-f**)

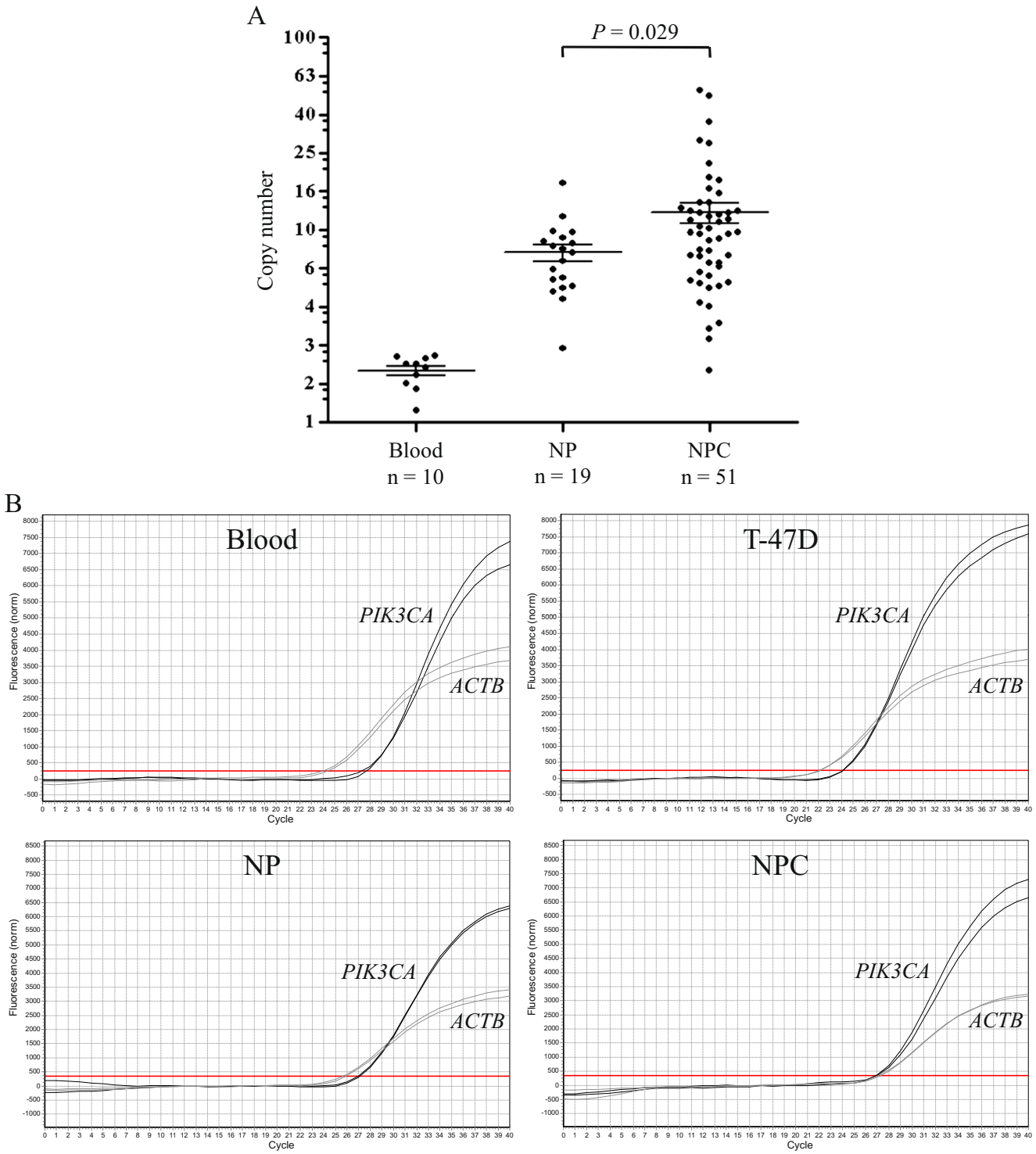

Fig. 2 Evaluation of *PIK3CA* gene copy number by quantitative real-time PCR in 51 NPC cases, 19 non-malignant nasopharynx tissues (NP), and 10 normal human whole blood samples. **a** Mean ± SD of copy numbers is shown. Statistical analysis was done using unpaired t test with Welch's correction. **b** Representative of PCR amplification curves generated for the *PIK3CA* and *ACTB* genes in a NPC sample with *PIK3CA* amplification, a NP tissue without *PIK3CA* amplification, the T-47D breast cancer cell line (positive control with 5 copies), and in a normal human blood sample (calibrator). Each test was performed in duplicate and is shown by overlapping curves. *ACTB* was used as the endogenous reference gene and the *PIK3CA* copy number relative to calibrator was calculated as described in the Materials and Methods section

all the statistical analyses, except the Freeman–Halton extension of Fisher's exact test, which was performed using VassarStats online software (Vassar College, Poughkeepsie, NY; http://vassarstats.net/index.html). A two-

sided *P*-value of less than 0.05 was considered statistically significant.

Results

We performed an immunohistochemical staining for p110α catalytic subunit of PI3K in 74 NPC tissues. Immediately adjacent normal-appearing nasopharyngeal mucosa was found in 42 of these NPC tissues. The mean rank score of PI3K p110α expression in tumors was significantly higher than that in the adjacent normal nasopharyngeal mucosa ($P < 0.001$; Fig. 1). To define the frequency of PI3K p110α overexpression, tissues with a score of 2+ or 3+ were considered to have overexpression. Data demonstrated that 44.6 % (33/74) of NPCs and 4.8 % (2/42) of the normal nasopharyngeal mucosa overexpressed PI3K p110α protein.

We next investigated whether the PI3K p110α overexpression could be attributed to the copy number gain of its gene, *PIK3CA*. Using quantitative real-time PCR, the *PIK3CA* copy number was successfully evaluated in only 51 out of 74 NPC tissues due to some invalid results or insufficient samples. The copy number of *PIK3CA* in 10 samples of normal human whole-blood DNA ranged from 1.16 to 2.21 with the mean of 1.85 ± 0.35 copies (Fig. 2). Relative to normal blood samples, the copy number in 51 NPC and 19 non-malignant nasopharynx tissues was calculated, demonstrating the mean of 12.29 ± 10.50 and 7.64 ± 3.32 copies, respectively. The mean *PIK3CA* copy number in NPC cases was significantly higher ($P = 0.029$) than that in non-malignant nasopharynx tissues. To determine the frequency of gene amplification or copy number gain of *PIK3CA* in NPC, mean + 2SD (standard deviation) of copy numbers in the non-malignant nasopharynx tissues was taken as cut-off value (14.28 copies). Data demonstrated that 19.6 % (10/51) of NPCs had *PIK3CA* copy number gain. Moreover, we found a significant correlation between the *PIK3CA* copy number and the mean rank score of PI3K p110α expression in NPC ($P = 0.020$, $r = 0.324$; Table 1). We have previously examined the Akt phosphorylation status by immunohistochemistry in some of the NPC specimens used in the current study [12]. By using the immunoscoring data, statistical analysis showed that the Akt phosphorylation on Ser473 or Thr308 site was not significantly correlated with *PIK3CA* copy number or PI3K p110α expression (Table 1).

We next analyzed whether the *PIK3CA* amplification and PI3K p110α expression in NPC tissues were associated with patient and tumor variables including gender, age at diagnosis, race, and histological type. However, no significant association was observed (Table 2).

Table 1 Correlations among *PIK3CA* copy number, PI3K p110α expression, and Akt phosphorylation in NPC tissues

		PIK3CA copy number	p-Akt (Ser473)	p-Akt (Thr308)
PI3K p110α expression	r	0.324	0.201	−0.135
	P	0.020	0.089	0.401
	n	51	73	41
PIK3CA copy number	r		0.213	0.107
	P		0.137	0.644
	n		50	21

Spearman's rank correlation test. r, correlation coefficient; n, number of samples was less than the total sample size ($n = 74$) due to the limited data available for analysis or some samples being not assessable

Discussion

Our previous study [12], in concordance with a report [16], shows no *PIK3CA* hotspot mutations in NPC tissues based on the mutational analysis of exons 9 and 20 of *PIK3CA* gene. In the current study, we investigated an alternative molecular alteration in the *PIK3CA* gene by assessing the expression of its encoded protein, the p110α catalytic subunit of PI3K (PI3K p110α). Immunohistochemical staining showed a

Table 2 Associations of *PIK3CA* copy number and PI3K p110α expression with patient and tumor variables

Characteristic	*PIK3CA* copy number[a]				PI3K p110α expression		
	n	No gain	Gain	P	n	MR[c]	P
Gender							
Male	37	30	7	1.000[d]	57	38.72	0.341[f]
Female	14	11	3		17	33.41	
Age (years)							
≤ 50	16	15	1	0.142[d]	27	36.59	0.769[f]
> 50	35	26	9		47	38.02	
Race[b]							
Chinese	26	19	7	0.294[d]	37	35.39	0.484[f]
Non-Chinese	24	21	3		36	38.65	
Histological type							
SCC (type I)	6	6	0	0.323[e]	10	37.50	0.762[g]
NKC (type II)	24	20	4		38	39.04	
UC (type III)	21	15	6		26	35.25	

SCC (type I), squamous cell carcinoma; NKC (type II), non-keratinizing carcinoma; UC (type III), undifferentiated carcinoma

[a] Copy number gain of *PIK3CA* is defined here as copy number of >14.28

[b] The race of one case was unavailable in the patient record

[c] MR, mean rank

[d] Fisher's exact test

[e] Freeman–Halton extension of Fisher's exact test

[f] Mann–Whitney U-test

[g] Kruskal–Wallis test

significantly higher level of PI3K p110α in NPC (44.6 % of cases), compared to the normal nasopharyngeal mucosa (4.8 %). To the best of our knowledge, this is the first report on overexpression of PI3K p110α protein in NPC. Aberrant PI3K p110α expression has also been reported in other cancers, such as breast [21], colorectal [22], head and neck [23], esophageal [24], ovarian [25, 26], and pituitary [27] cancers.

We next assessed the *PIK3CA* gene copy number by using quantitative real-time PCR to explore whether the PI3K p110α overexpression resulted from *PIK3CA* gene amplification. We found *PIK3CA* amplification in 19.6 % of the NPC cases, which is comparable to the reported frequency at 21.6 % in Tunisian NPC patients [17]. The PI3K p110α protein expression, however, was not evaluated in the Tunisian NPC. In the current study, we observed a positive correlation between *PIK3CA* copy number and PI3K p110α immunostaining, suggesting that the gain of *PIK3CA* copy number translates into increased expression of the corresponding protein, PI3K p110α. This observation is consistent with the previous studies on other cancers [23, 26, 27]; however, no significant correlation has also been reported [24, 25]. This discrepancy might be due to the modulation of the protein level by transcriptional, post-transcriptional or post-translational mechanisms. For example, the transcription of *PIK3CA* has been shown to be regulated by the tumor suppressor protein p53, the oncoprotein Y-box binding protein-1, forkhead box O3a, and nuclear factor-kappa B [28, 29].

To investigate the functional implications of *PIK3CA* amplification and PI3K p110α overexpression, we tested whether these molecular aberrations may be linked to the activating phosphorylation of Akt. Our results, in line with those from previous studies [26, 30, 31], did not show correlation of *PIK3CA* amplification or PI3K p110α overexpression with increased Akt phosphorylation. Although this correlation has been reported previously [10, 25, 32], our results hint that other downstream effectors of PI3K, such as the serum and glucocorticoid-regulated kinase (SGK) family [3, 33], may contribute to NPC pathogenesis and therefore, are worthy of further investigation.

PI3K p110α overexpression and *PIK3CA* amplification were reported to be associated with poor prognosis in breast cancer and Tunisian NPC patients, respectively [17, 21]. Unfortunately, the TNM classification and patient survival data were not available for the current study to support these findings. Our data demonstrated no association of *PIK3CA* amplification or PI3K p110α overexpression with patient and tumor variables including gender, age at diagnosis, race, and histological type.

PI3K p110α may be a promising target for novel therapeutic strategies in NPC. In recent studies, PI3K or dual PI3K/mTOR inhibitors have shown efficacy in tumor growth inhibition, chemo/radiosensitization, and anti-metastasis in NPC xenograft models [34–36]. In summary, our current study demonstrates that PI3K p110α overexpression, which may be attributed, at least in part, to the *PIK3CA* amplification, plays an important role in the molecular pathogenesis of NPC. This molecular information supports the application of PI3K pathway-targeted therapy as a rational treatment in NPC. However, the downstream molecular mechanisms of PI3K remain to be elucidated. Further studies with larger sample size and clinicopathological features are required to add more conclusive data that can provide prognostic prediction and therapeutic implications in NPC.

Acknowledgments This study was supported by the Malaysian Ministry of Science, Technology and Innovation (Grant No. 02-01-04-SF1312) and the UPM Research University Grants (No. 04-01-11-1178RU).

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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