The Prevalence of PIK3CA Biomarkers in Tumor Microenvironment of Human Non-Small Cell Lung Cancer

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Abstract: Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases involved in many cellular processes, including cell growth, proliferation, differentiation, motility and survival, that can inhibit therapy profoundly. NSCLC accounts for approximately 80% of all lung cancers and is further subtyped into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Somatic oncogenic alterations can further molecularly subdivide these NSCLC subtypes. The general objective of the current work was to study the prevalence of PIK3CA expression and mutations in tumor microenvironment of human NSCLC. Thirty-three paraffin blocks were utilized from NSCLC archival cases for further investigations by using immunohistochemical staining and PCR/sequencing techniques for examination of PI3KCA cellular expression and detection of mutation in exons 9 and / or 20 of PI3KCA, respectively. Immunohistochemical results showed that PIK3CA protein was localized in the cytoplasm of NSCLC as brown granules. The rating of scores of PIK3CA immunostaining of archived NSCLC revealed that twenty six cases (78.8%) showed positive immunohistochemical staining while seven cases (21.2%) showed negative immune staining. PCR/ sequencing results of exon 9 revealed six substitution mutations and three indels (insertion / deletion) mutations were observed in the alignment. It was found that all the observed SNP positions were novel except 74681. The novelty of each observed SNP was detected using the NCBI SNP detector and it was found that all the observed SNP positions were novel except 7468. PCR/ sequencing results of exon 20 revealed that the alignment results of all the patient samples for exon 20 revealed the presence of SNPs in variants no. 3, 5 and 7. All of these variations were only substitution mutations of this PCR amplicons. The exact position of each observed mutation was mentioned in the NCBI reference sequences. The data in hands shows that PIK3CA-mutated NSCLC is a clinically and genetically heterogeneous group. This holds true for the AD and SQCC subgroups and strongly suggests that PIKCA mutations do not define a distinct lung cancer subgroup amendable to specific therapy. Rather, these mutations seem to represent passenger mutations widely distributed among the other genetically defined subgroups. The status PIK3CA mutation may serve as a prognostic factor of poor survival.

Key words: PIK3CA · Tumor Biomarkers · Tumor Microenvironment · Non-Small Cell Lung Cancer · NSCLC

INTRODUCTION

Lung cancer is among the most deadly cancers for both men and women [1]. Its death rate exceeds that of the three most common cancers (colon, breast and pancreatic) combined [2]. Lung cancer has poor prognosis; over half of people diagnosed with lung cancer die within one year of diagnosis and the 5-year survival is less than 18% (around 17.8%). There are two main subtypes of lung cancer, small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC), accounting for 15 and 85% of all lung cancer, respectively [3].

Non-small cell lung cancer (NSCLC) remains the most widely recognized reason for cancer related demise in the western world. NSCLC accounts for approximately 85% of all lung cancers. Histologically, NSCLC is divided

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into adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma. Genetic and epigenetic alterations have been distinguished as often as possible in lung cancer, for example, promoter methylation, gene mutation and genomic amplification. In any case, the collaboration amongst genetic and epigenetic occasions and their essentialness in lung cancer genesis remains poorly understood. Patients with NSCLC require a complete staging workup to evaluate the extent of disease, because stage plays a major role in determining the choice of treatment [4, 5].

The most common type of NSCLC is adenocarcinoma; it comprises around 40% of all cancers. Squamous cell carcinoma (SCC) accounts for 25-30% of all cases. Large cell (undifferentiated) carcinoma accounts for 5-10% of lung cancers. All subtypes are strongly correlated with cigarette smoking [4]. Adenocarcinoma is the most common type of lung cancer in smokers and nonsmokers in men and women regardless of their age. It tends to occur in the periphery of the lung, which might be due to the addition of filters in cigarettes preventing large particles from entering the lungs. This results in deeper inhalation of cigarette smoke, leading to peripheral lesions [6]. Compared to other types of lung cancer, adenocarcinoma tends to grow slower and has a greater chance of being found before it has spread outside of the lungs [7].

Personalized medicine by targeting appropriate molecular markers in tumors has helped to improve survival in patients with NSCLC. There are targeted agents that have been successful against epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements. Through genomic testing, other molecular changes have been found including gene rearrangements of ROS1 and RET, amplification of MET and activating mutations in *BRAF*, *HER2* and *KRAS* genes, which might be potential targets for future therapies. Extra biomarkers were examined for application in cancer treatment include the PIK3CA, HER2, BRAF, ROS, RET, NRAS, MET and MEK1. As research proceeds with, more biomarkers are being found, each having the capacity to open up treatment choices that won't be existed previously. The disclosure of biomarkers is exhibiting the molecular complexity of NSCLC. No attempts are moving toward molecularly based analysis and treatment of these cancers [8, 9].

PI3Ks are a group of lipid kinases that phosphorylate the 3'-hydroxol aggregate in phosphatidylinositol and phosphoinositides [10]. There are three classes of PI3K (I-III), which are ordered by structure and substrate specificity. They are heterodimeric proteins with catalytic and regulatory subunits [11]. Phosphoinositides are universal in the cellular membrane of many types of cell and tissue and are important second messengers for intracellular signaling in few procedures. Of the enzymes required in creating particular phosphoinositides, a group of lipid kinases, that is altogether known as the PI3Ks. Despite the fact that the PI3Ks have especially vital capacities in the immune system, it has been hard to assess the part of individual PI3Ks in cellular immune responses owing to an absence of inhibitors particular for each PI3K [12, 13]. The aim of the study was to study the prevalence of PIK3CA expression and mutations in tumor microenvironment of human non-small cell lung cancer.

MATERIALS AND METHODS

Patients and Specimens: The underlying study was designed to investigate the expression and mutation in PIK3CA in NSCLC. Out of 130 cases diagnosed as NSCLC at the Department of Pathology, Faculty of Medicine, University of Alexandria, Egypt, during the period of month?? 2013 to month?? 2015, only thirty-three paraffin blocks were sufficient for further investigations by using immunohistochemical staining and PCR techniques for detection of mutation in exons 9 and / or 20 of PI3KCA, utilizing PCR and sequencing technique.

NSCLC tumor samples were recruited from archived cases of Department of pathology and data concerning cases were collected. Thirty three paraffin-fixed NSCLC tissue and five control samples were available from the archival cases in the Department of Pathology, Faculty of Medicine, University of Alexandria, Egypt. Control samples were obtained from safety margins of the tumors. Histological review of all lung specimens was performed by two independent pathologists and tissue, related to areas of interest, were scrapped into 1.5 ml sterile microcentrifuge tubes. Evaluation of the expression of PIK3CA/PI3K in lung tissue was performed by using immunohistochemical method.

Immunohistochemical Staining and Assessment: For PIK3CA immunohistochemistry, 4-μm sections were cut from the paraffin blocks and transferred onto coated slides. The sections were deparaffinized through incubation in a dry oven at 60? for 1 hour and three washes in xylene. Following blockage of the endogenous peroxidase activity using 5% hydrogen peroxide in methanol for 15 minutes at 37?, the sections were autoclaved in a pH-6.0 epitope retrieval solution for

20 minutes for antigen retrieval. The sections were stained with a 1:1000 dilution of PI3K mouse anti-Human monoclonal (4F3) antibody. Following incubation in a humidified chamber at 4? for 16 hours, a secondary antibody treated using the Econon Tek HRP Anti-Polyvalent (DAB) Detection kit (ScyTek Laboratories, Inc. Logan, USA). Diaminobenzidine was used as chromogen for color development. PIK3CA immunostaining was evaluated by the pathologist. Two independent blinded observers evaluated the expression of PIK3CA. Cytoplasmic granular staining of PIK3CA was considered as positive immunoreactivity. Adjacent non-tumorous tissue, in the tumor core or excision biopsies, was used as negative controls. External normal control sections from safety margins of 5 cases were also taken. PIK3CA expression was graded based on the proportion and intensity of staining. The stained cell proportion was scored as: 0 ($\leq$ 5%); 1 (>5% to $\leq$ 25%); 2 (>25% to $\leq$ 50%); and 3 (>50%). Intensity of the immunostaining was scored as follows: 0 (negative); 1+ (weak positive); 2+ (moderate positive); and 3+ (strong positive). The final score was calculated by multiplying the proportion score with the intensity score. Final scores of <4 were considered negative, whereas scores of $\geq$ 4 were considered positive.

PIK3CA Mutation Detection: *DNA extraction*. Genomic DNA was extracted from 33 formalin-fixed and paraffin-embedded (FFPE) ESCC tissue samples with minimum of 75% malignant cells. Using QIAamp$^@$ Genomic DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration and purity of DNA were measured by a nanodrop (BioDrop μLITE, BioDrop Co., UK), while the DNA integrity was checked by a standard 1% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 μg/ml) in TAE (40 mM Tris acetate; 2 mM EDTA, pH 8.3) buffer, using a 1 kb ladder as a molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea). The isolated DNA was used as a template for PCR. Extracted genomic DNA was subjected to PCR/sequencing procedures for mutation detection in exons 9 and 20. The detailed procedures of Qiagen manufacturer for extraction were as follows:

PCR Amplification: Two total PCR fragments were selected for amplification. They were covered two unique nuclear DNA positions of the isolated genomic DNA of each sample including exon 9 and exon 20 of PIK3CA gene. A list of specific PCR primers, used to amplify two different portions of PIK3CA gene in the study, is illustrated in Table 1. The 261 bp and the 352 bp amplicons cover exon 9 and exon 20, respectively.

The exact positions of the studied PCR amplicons were graphically highlighted in the NCBI website with all intronic and exonic positions being identified. The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using AccuPower PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). Each 20μl of PCR premix was contained 1 U of Top DNA polymerase, 250 μM (μmole) of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program, shown in Table 2, was applied in PCR thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Daejeon, South Korea).

Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris–Borate, pH 8.3), using a 100-bp ladder (Cat # D-1010, Bioneer, Daejeon, South Korea) as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only one clean and sharp band in order to be submitted into sequencing successfully. The PCR mixture contained 50 ng of DNA, 5 pmol/L of each primer, 2.5 nmol/L of each dNTP and 1.25 U of Taq Gold DNA polymerase in 11.5 mL of buffer containing 0.04 mmol/L of Mg^{2+}.

PCR Amplicons Sequencing. In case of both exon 9 and exon 20, DNA amplicons were commercially sequenced from both ends (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not due to PCR or sequencing artifacts. The PIK3CA gene reference database (GenBank acc. NG_012113.2) were retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov). The position and other details of both retrieved PCR fragment was identified, as demonstrated in Table 3.

Interpretation of Sequencing: The sequencing results of the PCR products of different samples were edited, aligned and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variation was translated into amino acids in a reading frame

Table 1: A list of specific PCR primers used to amplify two different portions of PIK3CA gene in the study

| Primer code | Description | Accession Number | Amplicon Locus | | Amplicon Size ~ |
			Start	Stop	
*Exon9-F	ATCCAGAGGGGAAAAATATG	NG_012113.2	74630	74890	261
*Exon9-R	ATGCTGAGATCAGCCAAAT				
*Exon20-F	TCATTTGCTCCAAACTGACCAA		90530	90881	352
*Exon20-R	TGGAATCCAGAGTGAGCTTTCA				

*The 261 bp amplicons and the 352 bp amplicons covered exon 9 and exon 20, respectively

Table 2: RT-PCR Protocols for PIK3CA

	Exon 9	Exon 20
Number of Cycle	35	35
Pre-denaturation	95°C for 5 minutes	95°C for 5 minutes
Denaturation	95°C for 30 seconds	95°C for 30 seconds
Annealing	56°C for 40 seconds	58°C for 40 seconds
Extension	72°C for 45 seconds	72^0 C for 45 seconds
Final Extention	72°C for 7 minutes	72°C for 7 minutes

Table 3: The position and length of the two amplicons used to amplify portions in the PIK3CA gene in the study

No.	Genetic locus within PIK3CA gene	Genetic locus sequences (5' - 3')*	Length
1	Exon 9	ATCCAGAGGGGAAAAATATGACAAAGAAAGCTATATAAGATAT TATTTTATTTTACAGAGTAACAGACTAGCTAGAGAC AATGAATTAAGGGAAAATGACAAAGAACAGCTCAAAGCAATTTCTACAC GAGATCCTCTCTCTGAAATCACTGAGCAGGAGAAAGATTTTCTATGGA GTCACAGGTAAGTGCTAAAATGGAGATTCTCTGTTTCTTTTTCTTT ATTACAGAAAAAATAACTGAATTTGGCTGATCTCAGCAT	261
2	Exon 20	TCATTTGCTCCAAACTGACCAAACTGTTCTTATTACTTATAGGTTTC AGGAGATGTGTT ACAAGGCTTATCTA GCTATTCGACAGCATGCCAATCTC TTCATAAATCTTTTCTCAATGATGCTTGGCTCTGGAATGCCAGAACTAC AATCTTTTGATGACATTGCATACATTCGAAAGACCCTAGCCTTAGA TAAAACTGAGCAAGAGGCTTTGGAGTATTTCATGAAACAAATGAAT GATGCACATCATGGTGGCTGGACAACAAAAATGGATTGGATCTTCC ACACAATTAAACAGCATGCATTGAACTGAAAAGATAACTGAGAAAA TGAAAGCTCACTCTGGATTCCA	352

*The highlighted regions in each amplicon referred to both forward and reverse primers that confined both amplicons. The sequence of the revere primer was mentioned in the reverse complement order

corresponds to the reference amino acid sequences using the Expasy online program (http://web.expasy.org/translate/). Multiple amino acid sequence alignment was made between the reference amino acid sequences and its observed variations using the Clustal Omega program from the UniProt website (http://www.uniprot.org/align/).

To determine whether each observed single nucleotide polymorphism, SNP, is a novel, the NCBI-dbSNP website was used (https://www.ncbi.nlm.nih.gov/projects/SNP/). Each observed SNP was submitted to this program to identify whether this SNP is "known" or "novel".

RESULTS

The underlying study was carried out to investigate PIK3CA expression and mutation in DNA isolated from lung cancer in order to correlate with stages and prognosis of the disease. The archival studied cases belonged to patients with a minimum age of 27 years old and a maximum age of 84 years old. The median age was 63 years [range: 27-84] with a mean value of 60.55 ± 12.17. They were distributed according to gender to 72.7% males (24 out of the 33 cases) and 27.3% females (9 cases). Only five cases of patients (15%) were reported as smokers. According to histopathological types, majority of cases was diagnosed as squamous cell carcinomas (72.7%); of which 9.1% was well differentiated, G1, 33.3% was moderately differentiated, G2 and 30.3% was poorly differentiated, G3. Adenocarcinoma was diagnosed in 21.2% of cases; of which 6.1% was well differentiated, G1, 12.1% was moderately differentiated, G2 and 3% was poorly differentiated, G3. Two cases (6.1%) were undifferentiated carcinoma.

Immuno his to Chemistry: The rating of scores of PIK3CA immunostaining of archived NSCLC revealed that twenty six cases (78.8%) showed positive immunohistochemical staining while seven cases (21.2%) showed negative immune staining (Figures 1 to 3). Scoring of immunohistochemical results were performed as follows; The stained cell proportion was scored as: 0 ($\leq$5%); 1 (>5% to $\leq$25%); 2 (>25% to $\leq$50%); and 3 (>50%); Intensity of the immunostaining was scored as follows: 0 (negative); 1+ (weak positive); 2+ (moderate positive); and 3+ (strong positive); The final score was calculated by multiplying the proportion score with the intensity score; and Final scores of <4 were considered negative, whereas scores of $\geq$ 4 were considered positive. The scoring results of immunohistochemical staining ranged from 0.0-6.0 with a mean value of 4.33±1.41. No significant differences were detected between histopathological grading, types and the results of the immunostaining at p $\leq$ 0.05.

PI3K protein was localized in the cytoplasm of NSCLC as brown granules. Three cases of well-differentiated squamous cell showed positive immune staining and 10 out of 11 cases of moderate differentiated squamous cell carcinoma showed positive immunostaining while 7 out of 10 cases of poorly differentiated squamous cell carcinoma were positive for immunostaining. One out of the 2 cases of well-differentiated adenocarcinoma was positive for immunostaining, 3 out of 4 cases of moderate differentiated adenocarcinoma were positive for immunostaining and the poorly differentiated case was also positive for immunostaining. One out of the two cases of undifferentiated carcinoma was positive for immunostaining. No significant relation was found between the histological grading type and immune staining. Figures 1 to 3 illustrate some examples of the immunohistochemical staining of different NSCLC tissue.

PIK3CA DNA Sequencing and Mutation Detection: Genomic DNA was extracted from 33 samples of archival paraffin blocks of NSCLC and 5 controls of non-neoplastic tissue isolated from safety margins. The mean concentration of extracted DNA was of 18.1 ng/ml with a standard deviation of 2.4 ng/ml. DNA Purity (ratio of absorption at λ_{nm}?to?λ_{nm}?) ranged from 1.7 to 2.15 with a mean of 1.95 and a standard deviation of 0.4. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is

appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. DNA integrity was checked by a standard 1% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 μg/ml) in TAE buffer, thus bands of extracted genomic DNA are clearly presented.

Presence of mutations at exon 9 and exon 20 in DNA was investigated by sequencing. All analyses were repeated at least twice. Sequencing for PCR amplification products of exon 9 and exon 20 was performed using automated, dideoxy sequencing (sequencer Abi Prism 3100, Perkin-Elmer, Oak Brook, IL).

Through PCR-sequencing experiments for the amplified exons 9 and 20, several single nucleotide polymorphisms, SNPs, were discovered in both fragments of the two studied exons compared to control cases. The discovered SNPs were detected in ten out of twenty four squamous cell carcinoma (41.7%; 8 cases for exon 9 (33.3%) and 2 cases for exon 20 (8.3%), in four out of seven adenocarcinoma (57%; 2 cases for exon 9 and 2 cases for exon 20, 28.5% each) and in two out of two undifferentiated carcinoma (100%; 1 case for exon 9 and 1 case for exon 20, 50% each). The vast majority of *PIK3CA* mutations (70%) affected exon 9. This PCR-direct sequencing strategy was actually performed to identify the correlation between the observed SNPs in the patients with controls. This procedure was relied on to identify – if present – the causal SNP(s) for this disease. This study has discovered several point mutations in three genetic loci. These alterations were varied in their nature from substitutions, to indels (insertion – deletion mutations) but all of them didn't affect the reading frame of the resulting PIK3CA protein sequences; in other words, some of the observed SNPs were intronic while others were exonic in nature. But the later SNPs are silent and didn't change any amino acid in the resulting PIK3CA protein.

PIK3CA EXON 9: The first studied PCR amplicon is 261 bp, which partially covers intron 9 from 5' portion and exon 9 from the 3' portion. This fragment codes only 42 amino acids of exon 9. The PCR product for exon 9 was verified using agarose gel with a DNA marker at band of 261 bp. The alignment results of all the patient samples for exon 9 revealed the presence of a consensus sequences that are common among all these samples. It was very obvious for all the aligned sequences that all patients share exactly the same variations degree. These variations were ranged from 48 bp to 65 bp of this PCR amplicons.
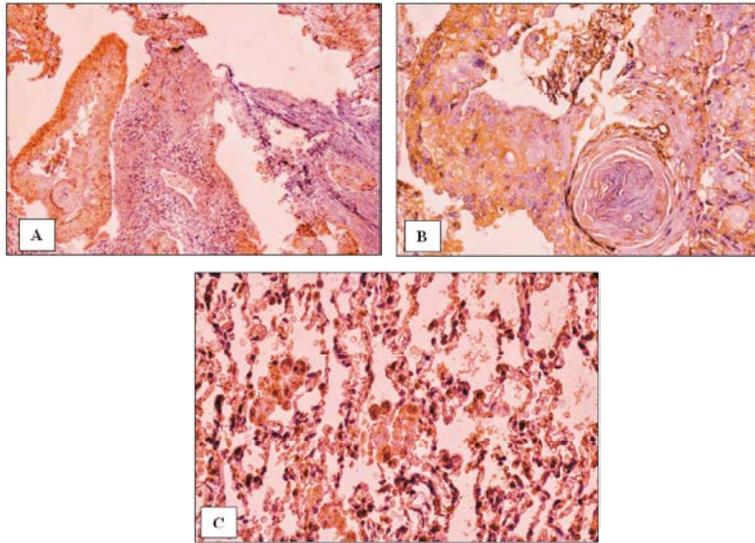
Fig. 1: Immunohistochemical staining of NSCLC archival tissue A; A case of w.d.sq.c.c. with pearl cell nests with pronounced staining at the outer layer (PIK3CAx100). B; HPV of the previous case showing mild positive brown cytoplasmic staining of less than 50% of tumor cells (PIK3CAx400). C; HPV of normal lung alveoli showing negative staining of the alveolar lining and positive staining of the macrophages (PIK3CAx400)
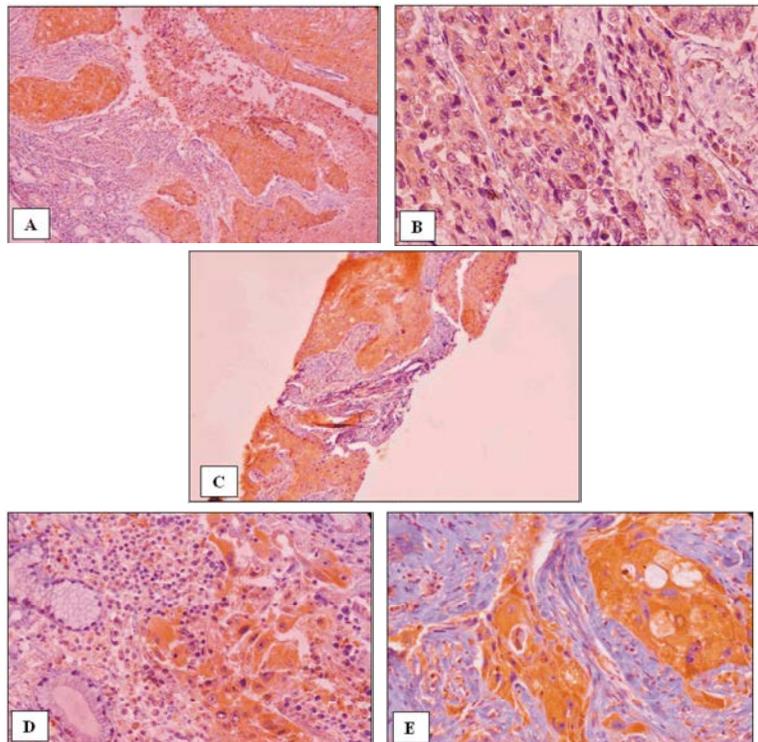

Fig. 2: Immunohistochemical staining of NSCLC archival tissue. A; a case of mod. sqcc showing severe cytoplasmic staining of most of tumor cells, score 6, (PIK3CAx100). B; HPV of the previous case showing diffuse cytoplasmic staining of the neoplastic cells (PIK3CAx400). C; another case of mod. sqcc showing severe cytoplasmic staining of most of tumor cells, score 6, (PIK3CAx100). D; HPV showing moderate staining intensity of neoplastic cells and negative staining of normal mucin secreting glands (internal control) (PIK3CAx400). E; another HPV showing severe staining intensity (PIK3CAx400)
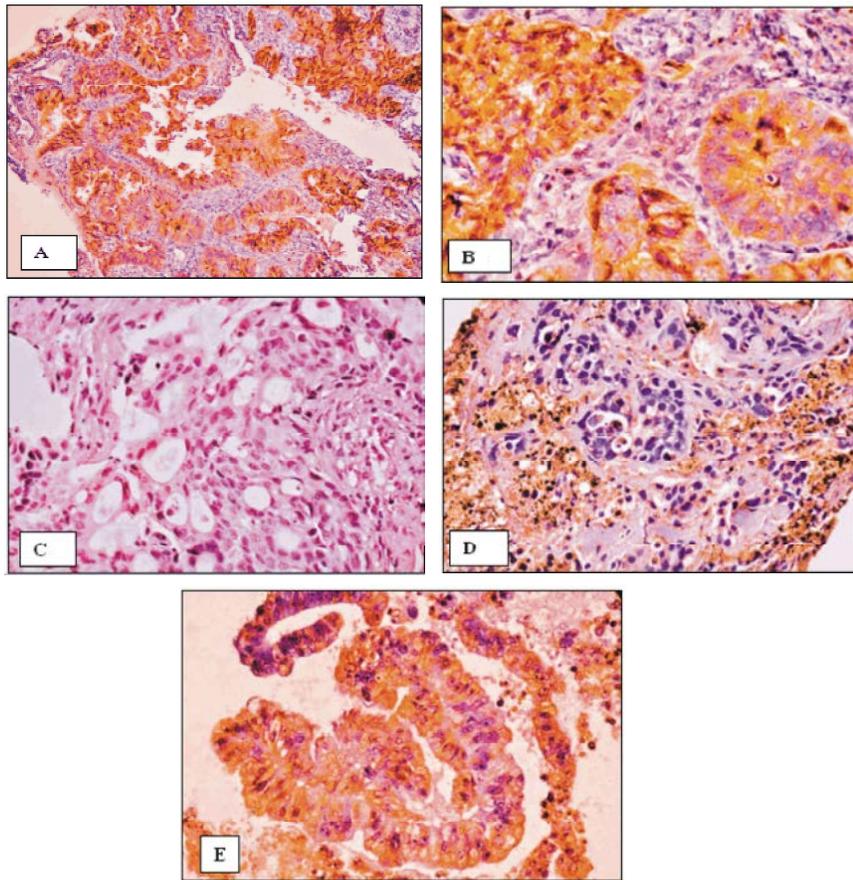
Fig. 3: Immunohistochemical staining of NSCLC archival tissue. A; a case of w. d. adenocarcinoma showing diffuse staining of all the neoplastic glands (PIK3CAx100). B; HPV of the previous case showing moderate staining intensity (PIKC3x400). C; a case of mod d. adenocarcinoma showing mild staining intensity (PIKC3x400). D; a case of mod d. adenocarcinoma showing negative staining reaction (PIK3CAx400). E; a case of papillary adenocarcinoma showing moderate staining intensity (PIK3CAx400)

The alignment was performed according to EditSeq DNA STAR software. Six substitution mutations and three indels (insertion / deletion) mutations were observed in the alignment. There were three types of variations dicovered in this fragment. The variation included three insertion mutations, three deletion mutations and six substitution mutations, as illustrated in Figure 4. Regarding the healthy control samples (var-8 to var-10), one type of nucleotide variation, which is substitution SNP was observed in var-10, as shown in Figure 4. To determine whether each observed SNP is synonymous or non-synonymous, the uniprotKB was utilized in this aspect. After the alignment of the coding portion of the seven variants with their corresponding reference sequences, no amino acid change was observed, which means non-synonymous SNP was revealed from this alignment. A non-synonymous substitution is a nucleotide mutation that alters the amino acid sequence of a protein. It is contrasted with synonymous substitution which does not alter amino acid sequences. As non-synonymous substitutions result in a biological change in the organism, they are subject to natural selection. If a gene has lower levels of non-synonymous than synonymous nucleotide substitution, then it can be inferred to be functional gene because a K_a/K_s ratio<1 is a hallmark of sequences that are being constrained to code for proteins. Eventually, to sum up all the results obtained from the sequenced exon 9 fragment, the exact position of each observed mutation was mentioned in the NCBI reference sequences. However, no non-synonymous SNP was observed in the exon 9 and all the exonic SNPs are synonymous. The novelty of each observed SNP was detected using the NCBI SNP detector and it was found that all the observed SNP positions were novel except 74681.
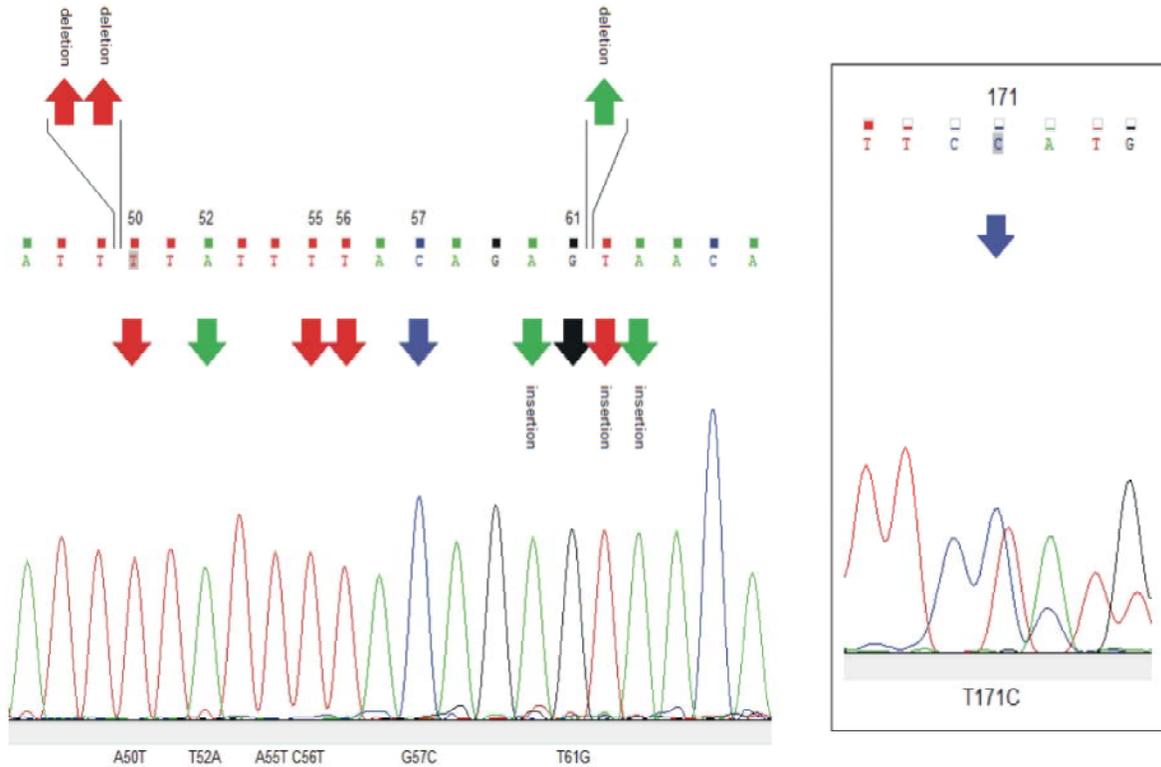
Fig. 4: The pattern of DNA chromatogram of all seven DNA variants of exon-9/PIK3CA gene (to the left hand side). Each substitution SNP was highlighted according to their positions in the PCR products (A50T, T52A, A55T, C56T, G57C, T61G), three deletion mutations (the above directed arrows) and three insertion mutations (the below directed arrows) were observed. To the right hand side, figure illustrates the pattern of DNA chromatogram of the healthy control var-10 DNA variants of exon-9/PIK3CA gene. The observed substitution SNP was highlighted according to its positions in the PCR products (T171C)

PIK3CA EXON 20: The other studied PCR amplicon is 352 bp, which partially covers intron 19 from 5' portion and exon 21 from the 3' portion. This fragment codes only 90 amino acids only from the exon 21, which constitutes the N-terminus of PIK3CA gene product. The PCR product for exon 20 was verified using agarose gel. The alignment results of all the patient samples for exon 20 revealed the presence of SNPs in variants no. 3, 5 and 7. All of these variations were only substitution mutations of this PCR amplicons. Both variants no. 3 and no. 5 included one substitution mutation, while the variant no. 7 has three substitution mutations. However, the nature and pattern of each observed SNP was shown in Figure 5, illustrating the pattern of DNA chromatogram of DNA variants of exon-20/PIK3CA gene. A, refers to the mutation observed in variant No. 3 (A340C). B, refers to the mutation observed in variant No. 5 (A340C). C, refers to the mutations observed in variant No. 7 (G202A, T301C and A315G). Each substitution SNP was highlighted according to their positions in the PCR products. To determine whether each observed SNP is synonymous or non-synonymous, the uniprotKB was utilized in this aspect. After the alignment of the coding portion of the three mutant variants (variant 3, 5 and 7) with their corresponding reference sequences, no amino acid change was observed, which means no non-synonymous SNP was revealed from this alignment. Eventually, to sum up all the results obtained from the sequenced exon 20 fragment, the exact position of each observed mutation was mentioned in the NCBI reference sequences. However, no non-synonymous SNP was observed in the exon 20 and all the exonic SNPs are synonymous. The novelty of each observed SNP was detected using the NCBI graphical SNP detector and it was found that all the observed SNP positions were novel except 90731.
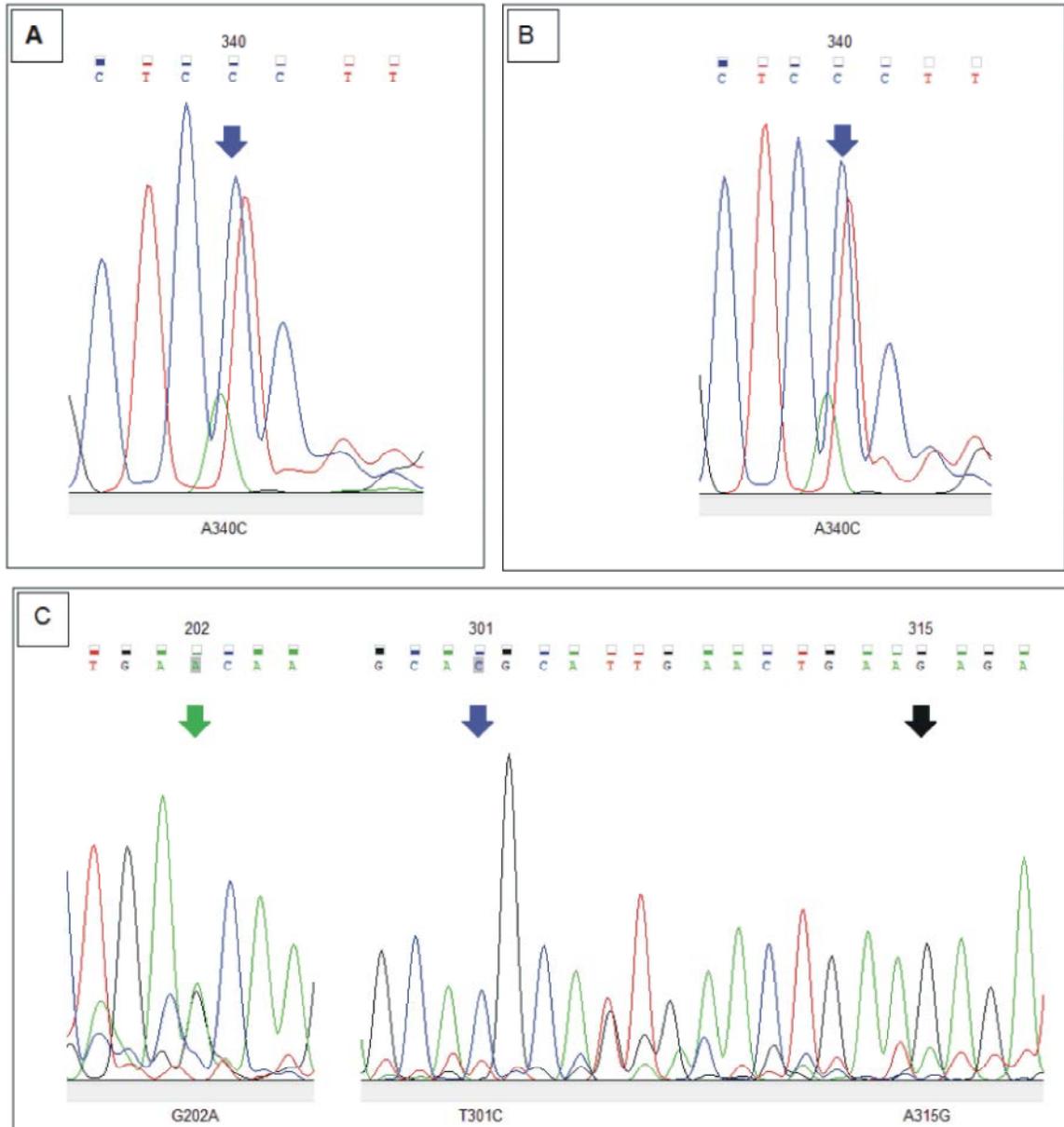
Fig. 5: The pattern of DNA chromatogram of DNA variants of exon-20/PIK3CA gene. A; refers to the mutation observed in variant No. 3 (A340C). B; refers to the mutation observed in variant No. 5 (A340C). C; refers to the mutations observed in variant No. 7 (G202A, T301C and A315G). Each substitution SNP was highlighted according to their positions in the PCR products

DISCUSSION

Non-small cell lung cancer (NSCLC) remains the most widely recognized reason for cancer related demise in the western world. Genetic and epigenetic alterations have been distinguished as often as possible in lung cancer, for example, promoter methylation, gene mutation and genomic amplification. In any case, the collaboration amongst genetic and epigenetic occasions and their essentialness in lung cancer genesis remains poorly understood [1].

Immuno His to Chemistry of PIK3CA: In the underlying study, PIK3CA immunohistochemistry over-expression occurred in 78% of the samples. Data are consistent with the previous studies where PIK3CA was present in 70%

of squamous cell carcinoma of lung cancer where, PI3K pathway activation in lung carcinoma was distinguished using p-S6, p-AKT and PTEN by evaluating of their expression through IHC [14]. However, these results were not consistent with previous studies on breast carcinomas, where PI3K over expression occurred in 25% of all tumor samples. Moreover, other studies informed that PI3K is not over expressed in melanocytic lesions [15]. High levels of p110δ expression have been documented in some solid tumor cell lines, but the functional role is unknown [16]. The current data showed no significant association between PI3-kinase expression and ages or histological types in lung cancer. Also, no relation between the *PIK3CA* gene mutations and histological types or grading was been found. There was a significant relation between *PIK3CA* gene copy number and age in lung carcinoma, which may be due to post-transcriptional, translational and protein degradation regulation which shows some genes are produced but not expressed on the cell surface [17].

PIK3CA Mutations: Through PCR-sequencing experiments for the amplified exons 9 and 20, several single nucleotide polymorphisms, SNPs, were discovered in both fragments of the two studied exons compared to control cases. The discovered SNPs were detected in ten out of twenty four squamous cell carcinoma (41.7%; 8 cases for exon 9 (33.3%) and 2 cases for exon 20 (8.3%), in four out of seven adenocarcinoma (57%; 2 cases for exon 9 and 2 cases for exon 20, 28.5% each) and in two out of two undifferentiated carcinoma (100%; 1 case for exon 9 and 1 case for exon 20, 50% each). The vast majority of *PIK3CA* mutations (70%) affected exon 9. This study has discovered several point mutations in three genetic loci. These alterations were varied in their nature from substitutions, to indels (insertion – deletion mutations) but all of them didn't affect the reading frame of the resulting PIK3CA protein sequences; in other words, some of the observed SNPs were intronic while others were exonic in nature. But the later SNPs are silent and didn't change any amino acid in the resulting PIK3CA protein.

A detailed characterization of PIK3CA-mutated NSCLC was provided in a large cohort study [18]. The prevalence of PIK3CA mutations was 3.7% confirming the published prevalence of about 2-4% in NSCLC [18-22]. Although mutations were found in AD and SQCC, the prevalence in SQCC was significantly higher with 8.9% compared to 2.9% in AD, thus exceeding the frequency

reported earlier for SQCC in smaller cohorts substantially. The frequency of adenocarcinoma in the cohort was 75.1% roughly representative for Caucasian NSCLC cohorts. However, squamous cell carcinoma was slightly underrepresented (15.6%). While this did not influence the results for the histology-dependent frequencies of PIK3CA mutations, their frequency in the total population of NSCLC patients might be slightly higher than 3.7% [18]. Comparison with a PIK3CA wild type cohort revealed a strong association with smoking. However, no further specific phenotypic characteristics concerning gender distribution, stage, metastatic spread or overall survival rate were found. The results of the current study are in consistent with the results of cohort study. The overall survival (OS) analyses showed no negative impact of PIK3CA mutations with the exception of EGFR-mutated patients treated systemically [18]. Noteworthy, the data cut-off was in 2012 and patients with EML4-ALK translocation treated systemically nowadays might have a better prognosis regarding OS than the 12.5 months observed in our group [22]. The vast majority of PIK3CA mutations (78.6%) affected exon 9 as described earlier. One exon 20 mutation was not described earlier that was detected in cohort study. Thus, screening of larger cohorts might reveal a more pronounced genetic heterogeneity in the future [18].

Co-occurrence of PIK3CA mutations with several driver mutations has been published previously for AD [21]. It was shown that SQCC *PIK3CA* mutations co-occur frequently with driver mutations namely affecting DDR2, KRAS, EGFR, BRAF, HRAS, NFE2L2, CTNNB1, MET and STK11, beside amplifications of FGFR1 and HER2 and gatekeeper mutations within TP53. Thus, in analogy to the missing specific phenotypic characteristics, these genotype analyses do not suggest a distinct genetic profile of PIK3CA mutated lung cancer neither in the AD nor in the SQCC subgroup [23]. With regard to optimization of molecular lung cancer diagnostics it is noteworthy that the frequency of co-occurring driver mutations was significantly higher using NGS technology (77.3%) compared to single-gene assay diagnostics (35.0%), as with NGS, a higher number of targets were screened simultaneously. This observation underlines the need of comprehensive molecular testing in order to better understand the molecular aberrations in lung cancer [19, 21, 23].

An unexpected observation was that *PIK3CA*-mutated NSCLC occurs significantly more often in patients with different prior malignances compared to

PIK3CA-wildtype NSCLC [21, 22]. In a cohort study, there was no specific association with distinct primary tumors, as these included various malignancies of different origin including frequent adenocarcinomas such as breast and colorectal cancer but also malignant lymphomas. Further understanding of this observation, which might represent an association with long-term carcinogenic cancer treatment effects as well as with an underlying genetic predisposition requires the analysis of germ line and primary tissue samples in larger cohorts [18]. It has been discussed controversially, whether PIK3CA mutations should be regarded as driver mutations thus representing potential targets for a specific blockade of this signal transduction pathway [18].

It was indicated that the finding of an association of SNPs with brain metastasis in patients with NSCLC may be biologically plausible. Patients carrying at least one variant allele in PIK3CA: rs2699887 had nearly twice the risk of brain metastasis as those without those variants. PIK3CA is the catalytic domain for PI3K, a known oncogene and is responsible for initiating signaling through this pathway, thus activating cell survival signals. Overexpression of PIK3CA was demonstrated in primary lung carcinomas and their metastases. Moreover, genomic amplification of PIK3CA in NSCLC is common, occurring in 70% of squamous cell carcinomas and 19% of adenocarcinomas. Increased PI3K activity would result in increased cell survival signals, causing increased metastases. Multiple mechanisms of PI3K activation may be responsible for the high levels of PI3K pathway activation, including PIK3CA mutations [24, 25].

In the underlying study, through PCR-sequencing experiments for the amplified exons 9 and 20, several single nucleotide polymorphisms, SNPs, were discovered in both fragments of the two studied exons compared to control cases. The vast majority of PIK3CA mutations (70%) affected exon 9, which is consistent with previous research. In fact, a major limitation of the underlying work is that it was not feasible to analyze the primary tumors genomically. Screening of larger cohorts might reveal a more pronounced genetic heterogeneity in the future related to NSCLC.

CONCLUSION

The underlying study revealed that further longitudinal studies are required to understand the pattern of mutations in PIK3CA related to NSCLC in Egyptian population, to correlate patterns with subtypes of NSCLC; AD and SQCC and to correlate the presence of mutations with resistant to treatment therapy.

REFERENCES

1. Siegel, R., J. Ma, Z. Zou and A. Jemal, 2014. Cancer statistics. CA Cancer J. Clin., 64(1): 9-29.

2. American Cancer Society, 2015. Cancer Facts and Figures Available online: http://www.cancer.org/acs/ groups/content/@editorial/documents/document/a cspc-044552.pdf.

3. National Cancer Institute, 2011. SEER Cancer Statistics Review, 1975-2011. Available online: http://seer.cancer.gov/csr/1975_2011/.

4. Zappa, C. and S.A. Mousa, 2016. Non-small cell lung cancer: current treatment and future advances. Transl Lung Cancer Res., 5(3): 288-300.

5. Sher, T., G.K. Dy and A.A. Adjei, 2008. Small cell lung cancer. Mayo Clin Proc., 83: 355-67.

6. Kenfield, S.A., E.K. Wei, M.J. Stampfer, B.A. Rosner and G.A. Colditz, 2008. Comparison of aspects of smoking among the four histological types of lung cancer. Tob Control, 17: 198-204.

7. Travis, W.D., L.B. Travis and S.S. Devesa, 1995. Lung cancer. Cancer, 75: 191-202.

8. Paez, J.G., P.A. Jänne, J.C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F.J. Kaye, N. Lindeman, T.J. Boggon, K.Naoki, H. Sasaki, Y. Fujii, M.J. Eck, W.R. Sellers, B.E. Johnson and M. Meyerson, 2004. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science, 304: 1497-1500.

9. Riely, G.J., J. Marks and W. Pao, 2009. KRAS mutations non-small cell lung cancer. Proc. Am. Thorac. Soc., 6: 201-205.

10. Engelman, J.A., J. Luo and L.C. Cantley, 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat. Rev. Genet, 7: 606-619.

11. Solomon, B. and R.B. Pearson, 2009. Class IA phosphatidylinositol 3-kinase signaling in non-small cell lung cancer. J. Thorac. Oncol., 4: 787-791.

12. Engelman, J.A., 2009. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer, 9: 550-562.

13. Vivanco, I. and C.L. Sawyers, 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer, 2: 489-501.

14. Singh, R.S., A.H. Diwan, P.S. Zhang and V.G. Prieto, 2007. Phosphoinositide 3-kinase is not overexpressed in melanocytic lesions. J. Cutan Pathol., 34(3): 220-225.

15. Barbara, A., M.D. Allison, B. Emily, G. Joseph, H. Erika, K.L. Blackwell, C. Livasy, K. Fritchie, A. Prat, J. C. Harrell, M.G. Ewend, L. A. Carey, C.R. Miller and C.K. Anders, 2011. Phosphatidylinositol 3-kinase pathway activation in breast cancer brain metastases. licensee BioMed Central Ltd, 13: R125.

16. Tzenaki, N., M. Andreou, K. Stratigi, A. Vergetaki, A. Makrigiannakis, B. Vanhaesebroeck and E.A. Papakonstanti, 2012. High levels of p110δ PI3K expression in solid tumor cells suppress PTEN activity, generating cellular sensitivity to p110δ inhibitors through PTEN activation. FASEB J., 26(6): 2498-2508.

17. Vogel, C. and E.M. Marcotte, 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet., 13(4): 227-232.

18. Scheffler, M., M. Bos, M. Gardizi, K. Konig, S. Michels, J. Fassunke, C. Heydt, H. Künstlinger and M. Ihle, 2015. PIK3CA mutations in non-small cell lung cancer (NSCLC): Genetic heterogeneity, prognostic impact and incidence of prior malignancies. Oncotarget, 6(2): 1315-1326.

19. Buettner, R., J. Wolf and R.K. Thomas, 2013. Lessons learned from lung cancer genomics: the emerging concept of individualized diagnostics and treatment. J Clin Oncol., 31(15): 1858-1865.

20. Oxnard, G.R., A. Binder and P.A. Janne, 2013. New targetable oncogenes in non-small-cell lung cancer. J. Clin Oncol., 31(8): 1097-1104.

21. Chaft, J.E., M.E. Arcila, P.K. Paik, C. Lau, G.J. Riely, M.C. Pietanza, M.F. Zakowski, V. Rusch, C.S. Sima, M. Ladanyi and M.G. Kris, 2012. Coexistence of PIK3CA and other oncogene mutations in lung adenocarcinoma-rationale for comprehensive mutation profiling. Mol. Cancer Ther., 11(2): 485-491.

22. Rekhtman, N., P.K. Paik, M.E. Arcila, L.J. Tafe, G.R. Oxnard, A.L. Moreira, W.D. Travis, M.F. Zakowski, M.G. Kris and M. Ladanyi, 2012. Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: lack of EGFR/KRAS and presence of PIK3CA/AKT1 mutations. Clin Cancer Res, 18(4): 1167-1176.

23. Sequist, L.V., R.S. Heist, A.T. Shaw, P. Fidias, R. Rosovsky, J.S. Temel, I.T. Lennes, S. Digumarthy, B.A. Waltman, E. Bast, S. Tammireddy, L. Morrissey, A. Muzikansky, S.B. Goldberg, J. Gainor, C.L. Channick, J.C. Wain, H. Gaissert, D.M. Donahue, A. Muniappan, C. Wright, H. Willers, D.J. Mathisen, N.C. Choi, J. Baselga, T.J. Lynch, L.W. Ellisen, M. Mino-Kenudson, M. Lanuti, D.R. Borger, A.J. Iafrate, J.A. Engelman and D. Dias-Santagata, 2011. Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice. Ann Oncol ESMO, 22(12): 2616-2624.

24. Yoo, S.S., J.E. Choi, W.K. Lee, Y.Y. Choi, S. Kam, M.J. Kim, H.S. Jeon, E.B. Lee, D.S. Kim, M.H. Lee, I.S. Kim, S. Jheon and J.Y. Park, 2009. Polymorphisms in the CASPASE genes and survival in patients with early-stage non-small-cell lung cancer. J. Clin Oncol., 27: 5823-5829.

25. Massion, P.P., P.M. Taflan, Y. Shyr, S.M. Rahman, P. Yildiz, B. Shakthour, M.E. Edgerton, M. Ninan, J.J. Andersen and A.L. Gonzalez, 2004. Early involvement of the phosphatidylinositol 3-kinase/Akt pathway in lung cancer progression. Am. J. Respir Crit Care Med., 170: 1088-1094.