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Genetic Alterations Profiling in North Macedonian Lung Cancer Patients

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Abstract

Objectives/background: Late diagnosis and inapropriately efficient treatment regimens of lung cancers lead to poor prognosis with 5-year survival rate of 22% for lung cancers in general (Non-Small Cell Lung Cancer (NSCLC) 26% and Small Cell Lung Carcinoma (SCLC) 7%). New targeted therapeutic agents and immunotherapeutics have to be developed and introduced only by first discovering new driver oncogenes and thorough investigation of the known ones. The aim of the current study is to investigate the prevalence of alterations in 4 most frequently altered genes in lung cancer - BRAF, EGFR, KRAS and PIK3CA and investigate their concomitant occurrence.

Methods: Real Time-Polymerase Chain Reaction (RT-PCR) was used to detect KRAS and EGFR mutations, multiplex PCR and microarray hybridization for KRAS/BRAF/PIK3CA mutations.

Results: In 603 consecutive lung cancer patients, simultaneous genotyping idenfied little less than 1/5 of the patients (n=110, 18.24%) with at least one genetic alteriation, of which six patients (1.00%) with two genetic alterations and no patient with more than two genetic alterations. 50 patients were identified with one or more KRAS mutations (8.29%), 45 patients with EGFR mutations (7.46%), PIK3CA mutations (1.82%) and BRAF mutation (0.66%). 50% of the co-occuring alterations were either on KRAS and PIK3CA genes (3/6), on KRAS and BRAF genes (2/6, 33.33%) or on EGFR and PIK3CA genes (1/6, 16.67%).

Conclusion: Large-scale testing for somatic alterations in EGFR, BRAF, KRAS and PIK3CA is feasible and impact therapeutic decisions in lung cancer management, and as such, should be routinely performed in order to generate a comprehensive genetic profile of lung cancers in a time and cost-effective manner.

Keywords: Lung cancer; BRAF; KRAS; EGFR; Gene alterations.

Introduction

Lung cancer incidence, survival rate & hystological properties

The World Health Organization (WHO)'s International Agency for Research on Cancer (IARC) GLOBOCAN cancer statistis 2022 reports lung cancer as the leading cause of cancer death, with an estimated 1.8 million deaths (18%) and is the most frequently diagnosed cancer worldiwide, with 2,480,675 new cases in 2022. The figures are sharply rising in female population and in emerging countries [1]. In North Macedonia in 2022, lung cancer was both most commonly diagnosed cancer and the leading cancer death, with 1150 new lung cancer cases, representing 15.2% of all new cancer cases diagnosed in 2022 and 1002 deaths [2].

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According to the National Comprehensive Cancer Network (NCCN) Guideline, 85-90% of lung cancers are caused by voluntary or involuntary (secondary) cigarette smoking [3]. Other possible risk factors include disease history (e.g., COPD), cancer history, family history of lung cancer, and expsoure to other carcinogens [4,5], including asbestos, arsenic, beryllium, cadmium, chromium diesel fumes nickel and silica [6,7]. The majority of patients either have metastatic disease at diagnosis or later experience a relapse. These patients benefit from palliative systemic therapies in terms of symptom control, quality of life and prolonged Overall Survival (OS). Histological subtypes of lung cancer are the cornerstone for selecting systemic therapies. The vast majority of lung cancers (approximately 80%) are NSCLC, including two major types [8]: (1) nonsquamous carcinoma - Large Celllung Carcinoma (LCLC), Large Cell Neuroendocrine Carcinoma (LCNEC), adenocarcinoma of lungs and/or bronchii, with or without methastases (e.g. on supraclavicular gland, liver, abdominal, ms ossei c. vertebrae, pleurae, cerebral) and lung cancer Not Otherwise Specified (NOS) and (2) squamous cell (epidermoid) carcinoma of the principal bronhii and/or lungs [9], while the remaining 20% are SCLC. Lung cancer entities are further refined by validation of recurrent oncogenic mutations as predictive biomarkers of therapeutically tractable oncogenic dependencies. This is of crucial importance, as lung cancer morphology exhibits broad spectrum and many tumors are atypical or lack the morphologic features necessary for improved different diagnoses. Here, comprehensive genomic analyses for several predictive and prognostic biomarkers and gene expression profiling are performed. The data obtained allow the differentiation of cancer subgroups based on molecular phenotypes [10], which is crucial for selection of treatment regimes.

Lung cancer treatment

Surgery, Radiotherapy (RT) and systemic therapy are the three modalities most commonly used to treat NSCLC patients, either alone or in combination, depending on the disease status. Surgical resection is the standard care for patients with stage I or II disease and for single-station non-bulky IIIA (N2, metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)) disease [11]. For IB (tumor ≥4 cm) to IIIA patients, it is usually followed by platinum-doublet chemotherapy, with very few trials showing the efficacy of adjuvant immunotherapy in stage IB to IIIA and several additional ongoing trials evaluating adjuvant oral TKI or immunotherapy [11]. RT, preferably Stereotactic Ablative Radiotherapy (SABR), is recommended for I-IV stage patients in different regimes; the doses and duration depend on the NSCLC stage (e.g. in locally advanced NSCLC; early-stage NSCLC with contraindications for surgery,e.g. major medical comorbidity, severely limited lung function; as preoperative or postoperative therapy, as therapy for limited recurrences and oligometastases or as palliative therapy for patients with incurable NSCLC) [12-15]. Standard systemic therapy for NSCLC is platinum-doublet chemotherapy, including cisplatin or carboplatin with permetrexed for non-squamous metastatic NSCLC, cisplatin or carboplatin or either paclitaxel or gemcitabine for squamous metastatic NSCLC [11] Comaprison of different platinum-based combinations showed no substantial differences in efficacy end-points and low overall Response Rate (RR) ranged between 17-32% [16]. The outcomes are underwhelming, as durable disease control is rarely achieved due to intrinsic or acquired drug resistance. For such patients, concurent or sequential target therapy of immunotherapy is cruical. However, approximately 85-90% of advanced NSCLC patients have no oncogenic alterations suitable for a targeted treatment and have poorer outcome and high rate of local relapse [17]. For them, treatment options include chemotherapy or single or combined immunotherapy [11]. Immunotherapeutic agents used in NSCLC are the Immune Checkpont Inhibitors (ICIs); three PD-1 inhibitors, cemiplimab-rwic, nivolumab and pembrolizumab and one PD-L1 antibody, atezolizumab is used in treatment of metastatic NSCLC [3]. Survival and duration of response increases as PD-L1 expression increases [11].

Driver oncogenes in lung cancer and targeted therapies

To date, there is no role for additional systemic therapy following adjuvant chemotherapy. The recent data regarding further adjuvant treatment with immunotherapy and Tyrosine Kinase Inhibitors (TKIs) have challenged the current standard of care [11]. Several molecular alterations, defined as driver mutations, have been identified as targets for which >20 targeted agents are developed and approved for treatment of advanced NSCLC patients. Such are the alterations in BRAF, Epidermal Growth Factor Receptor (EGFR) gene, Anaplastic Lymphoma Kinase (ALK) gene, Kirsten Rat Sarcoma virus (KRAS), (ROS-1) and Programmed (cell) Death-Ligand 1 (PD-L1) for squamous cell carcinoma, and rarely for mutation is phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha (PIK3CA) and Human Epidermal growth factor Receptor 2 (HER2). Additionally, identified key mechanisms of drug resistance in NSCLC show that additional mutations in tumor-driving genes may lead to drug insensitivity and tumor progression after treatment [17]. Usually, mutations/alterations are seen in a non-overlapping fashion, although between 1- 3% of NSCLC may harbor concurrent alterations [3].

NSCLC guidelines recommend testing for driver mutations in all non-squamous tumors. In fact, driver mutations are suspected in individuals who do not have a smoking history or those with a light smoking history, while tumors of squamous histology in non-smokers should be subject of testing on a case-by-case basis [11]. The international association for the study of Lung Cancer recommends testing for EGFR, ALK and ROS1 at a minimum; newer guidelines also recommend testing for BRAF, KRAS, MET, ERBB2 and RET [3,18], as well as ROS1 in all adenocarcinoma patients [18], BRAF in patients with metastatic non-squamous NSCLC or NSCLC NOS [3].

The aim of this non-randomized study is to investigated the gene expression and mutation signatures of different subtypes of lung cancers on 603 North Macedonian patients, including NSCLC (adenocarcinoma, squamous-cell carcinoma) and SCLC. Patients were examined for presence of genetic alterations in ≥2 genes: EGFR, BRAF, KRAS and PIK3CA.

Materials and methods

Patients and samples

This non-randomized, controlled, open-trial study included 603 patients with different types of lung cancers, treated at the University Clinic of Oncology and Radiology, UCC Skopje from November 17, 2010 to February 23, 2025. Tumor histology and stage were stimated according to WHO and pTNM classification according to the Union of International Cancer Control (UICC) [21]. All included patients had tissue samples taken at the Institute

of Pathology at the Faculty of Medicine, University Ss. Cyril and Methodius, Skopje, North Macedonia. All patients gave their written informed consent to participate in the study. The Ethics Committee for research with people at the Faculty of Medicine, UKIM in Skopje, North Macedonia approved investigation with human subjects according to The Code of Ethics of the World Medical Association- Declaration of Helsinki.

The age of the lung cancer patients enrolled ranged from 14 to 86 years (mean 62.56±9.34), of which 70.31% (n=424) are male and 29.68% (n=179) are female (Table 1). According to the histology, 99.17% (n=598) patients have NSCLC, and only 0.83% (n=5) have SCLC. Of the 598 NSCLC patients, 560 (92.87%) are diagnosed with adenocarcinoma, 27 (4.47%) are diagnosed with squamous-cell carcinoma and 11 (1.82%) are diagnosed with LCC. Metastatic NSCLC was diagnosed in 31 patients, 13 of which had metastases in lymph nodes (mediastinal, supraclavicular) alone, or together with hepatic or pulmonary metastases; brain metastases in 4 patients, liver and pleural metastases were present in 3 patients each, 2 patients had etiher metastses in mediastinum or bone, and 1 patient with abdominal metastasis, pul. bill and bone metastasis and pul. bill and liver metastasis. Other detailed characteristics of the patients are provided in Table 1.

DNA isolation

DNA isolation Form Paraffin Embedded tissue (FFPE) samples was performed using Cobas® DNA Sample Preparation Kit (Roche Diagnostics, Switzerland). DNA concentration was measured using ScanDrop2 (AnalyticJenna, Germany) spectrophotometer and dilution of isolated DNA was performed according to protocol for detecting mutations.

Detection of KRAS and EGFR mutations

Detection of EGFR mutations in exons 18-21 (exon 18: G719X (G719A, G719C, and G719S); exon 19: deletions and complex mutations; exon 20: S768I, T790M, and insertions and exon 21: L858R and L861Q) was performed using Cobas® EGFR Mutation Test V2 (Roche Diagnostics, Switzerland) on Cobas Z480 IVD RT-PCR (Roche Diagnostics, Switzerland). According to manufacturer's protocol, 2 ng/µL isolated DNA was used for detecting mutations. A mutant control and negative control are included in each run to confirm the validiy of the run. The Cobas® EGFR Test can detect mutations with at least 5% mutation level using the standard input of 50 ng per reaction well.

Detection of KRAS/BRAF/PIK3CA mutations

Detection of mutation in KRAS, BRAF, and PIK3CA genes are performed using KRAS/BRAF/PIK3CA Array which is based on a combination of multiplex PCR and microarray hybridisation. After isolation of DNA from FFPE samples, short multiplex PCR was used and after that hybridization and microarray technology was used to detect present mutations using Randox Evidence Investigator System (Randox Bioscience, UK). This system used innovative PCR priming technology which permits high discrimination between wildtype and mutant DNA. Providing there are enough copies of DNA present, approximately 1% of mutant can readily be detected in a background of wildtype genomic DNA. With this microarray assay can be detected BRAF V600E point mutation, 20 point mutations in codons 12, 13, 61, and 146 of KRAS gene, and three point mutations in PIK3CA gene (E542K, E545K, H1047R). For some samples, detection of V600E (1799T>A) mutation of BRAF gene was performed using Cobas® 4800 BRAF V600E Mutation IVD Test Roche Diagnostics, Switzerland) on Cobas Z480 IVD RT-PCR (Roche Diagnostics, Switzerland).

Results

The overall results from the genetic profiling of lung cancer patients are given in Table 2. 110/603 patients (18.24%) had at least one genetic alteration on one of the four examined genes (KRAS, BRAF, EGFR, PIK3CA). 432 patients were tested for presence of BRAF V600E mutation; the mutation was only found in 4 patients (0.93% of tested). Three of them are male and one female; three have adenocarcinoma and one squamous cell carcinoma. Further, 527 patients were tested for EGFR mutations and 45(8.54%) tested positive, 22 of which (48.89%) women and 23(51.11%) men, with average age of 61.36 years. All 45 patients were diagnosed with adenocarcinoma. Moret than half of them (24, 53.33%) had exon 19 deletion, and 14(31.11%) had the L858R mutation on exon 21. Exon 20 insertions were detected in 2 patients, while each of the following mutations was observed in only one patient: exon 19 indel, T790M, L816Q, exon 19 deletion + exon 20 insertion and exon 18 c.2177T>p.(Val726Gly). In addition, 193 patients were tested for KRAS mutations. Of them, 50(25.91%) had at least one KRAS mutation. 34(68%) were male and 16(32%) were female, with average age of 61.25 years. The majority of patients was diagnosed with adenocarcinoma (n=44, 88%), 2 with squamous-cell carcinoma and one with adenosquamous carcinoma and LCLC. The detected mutations included G12C (28%), codon 12/13 mutation (20%), G12D (14%), G12V (12%), G13C (4%), both G12D and G12C (4%), A146T (4%) and Q61H1, Q61L, Q6H2,

c.346>T p.(Gly12Cys), and co-appearance of G12D and G13D, codon 12/13 and G12C and G12D, G13D, Q61R and A146T, each detected in one patient (2%). Furthermore, 142 patients were tested for PIK3CA mutations. Of them, mutations were present only in 11(7.75%) of the patients; 6 male and 5 female, with average age of 66.91 years. 9 of the patients were diagnosed with adenocarcinoma, 1 with planocellular cancer and 1 with squamous cell cancer. 6 of the patients (54.55%) had E545K mutation, E542K was present in 3 patients (27.27%), while H1047R was present in 2 of them (18.18%).

Of the 603 consecutive lung cancer patients, simultaneous genotyping idenfied little less than 1/5 of the patients (n=110, 18.24%) with at least one genetic alteriation, of which six patients (1.00%) with two genetic alterations and no patient with more than two genetic alterations. 50 patients were identified with one or more KRAS mutations (8.29%), 45 patients with EGFR mutations (7.46%), PIK3CA mutations (1.82%) and BRAF mutation (0.66%). 50% of the co-occuring alterations were either on KRAS and PIK3CA genes (3/6), on KRAS and BRAF genes (2/6, 33.33%) or on EGFR and PIK3CA genes (1/6, 16.67%). All other details are given in Table 3.

Table 1: General data for the patients included in the clinical study.

Variable	n (%)
Gender	
Male	424(70.31%)
Female	179(29.68%)
Age	
mean ± SD	62.56 ± 9.34
< 39 years	17(2.82%)
40-49 years	41(6.80%)
50-59 years	111(18.41%)
60-69 years	216(35.82%)
70-79 years	205((34.00%)
≥ 80 years	13(2.16%)
Diagnosis	
NSCLC	598(99.17%)
Adenocarcinoma	560(92.87%)
Squamous-cell carcinoma	27(4.48%)
Large cell carcinoma	11(1.82%)
SCLC	5(0.83%)

Table 3: Demographics of patients with concomitant gene alterations.

Patient	Diagnosis	Gender	Age	Gene 2	Gene 2
P4	Adenocarcinoma	M	67	G12D, G12C KRAS	H1047R PIK3CA
P6	Adenocarcinoma	M	66	KRAS codon 12/13	BRAF V600E
P8	Carcinoma bronhii	M	68	G12C KRAS	BRAF V600E
P10	Adenocarcinoma	F	60	G12C KRAS	E542K PIK3CA
P16	Adenocarcinoma	F	61	L858R EGFR	H1047R PIK3CA
P20	Adenocarcinoma	M	67	G12D, G12C KRAS	E545K PIK3CA

Table 2: Demographics of examined genes and identified gene mutations.

Gene	Number of patients examined n (%)	No (% of patients with mutation	No (%) of patients with wild- type	Detected mutation	Numb er of patient's in which mutati on was detect ed	Gender		Average age of mutant patients	Avera ge age of wild- type pateints	Diagnosis of the patients			
						M	F			Adenoca rcinoma	Squamous-cell cancer	LCLC	SCLC
BRAF	432(71.52%)	4(0.93%)	428(99.07 %)	V600E	4	3	1	57.75	62.87	3	1	-	-
EGFR	527(87.40%)	45(8.54%)	482(91.46%)	Ex19 del	24	23	22	61.36	62.82	45(+2 planocellu lar carcinom a)	-	-	-
				Ex 19 indel	1								
				T790M	1								
				L858R	14								
				L861Q	1								
				Ex 20 ins	2								
				Ex 19 del+Ex 20 ins	1								
				Ex 18 c.2177T>6p. (Val72 6Gly)	1								
KRAS	193(31.95%)	50(25.91%)	143(74.09%)	G12C	14	34	16	61.25	64.25	44	2(+1 patient with adenosquamous cancer)	1	-
				Codon 12/13	10								
				G12D	7								
				G12V	6								
				G12D+G12C	2								
				G13C	2								
				Q61H1	1								
				Q61L	1								
				G12D+G13D	1								

				c.346>T p.(Gly12Cys)	1								
				Q6H2	1								
				A146T	2								
				Codon 12/13 and G12C	1								
				G12D+G13D+Q61R+A146T	1								
PIK3CA	142(23.51%)	11(7.75%)	131(92.25%)	H1047R	2	6	5	66.91	61.86	9(+1 patient with planocellular cancer)	1	-	-
				E545K	5								
				E542K	3								

Discussion

An individualized treatment approach to NSCLC starts with an accurate pathological diagnosis, imaging methods, endoscopic techniques for tissue sampling and staging according to pTNM classification, as well as establishing the correct tumor genotype. Comprehensive genomic analyses and functional preclinical validation are essential for expanding the potentially actionable genomic aberrations. According to current guidelines, NSCLC patients are screened for treatable oncogenic alterations for the purpose of proper treatment selection. Therefore, the goals of the genomic characterization of our NSCLC patients are to help guide therapy and ultimately lead to improved outcomes in patients with specific genomic changes. The patients from our study were tested for the following gene expression alterations, in descending order: EGFR (87.07%), BRAF (71.52%), KRAS (31.95%) and PIK3CA (23.51%). Most of the patients had one or more KRAS mutations (8.28%), followed by EGFR mutations (5.13%), PIK3CA mutations (1.82%) and BRAF mutation (0.66%). We expect that this proportion should increase as the scope of genotype-specific clinical trias is expanding at our institution and other centers.

EGFR mutations

The identification of activating EGFR mutations, together with increased sensitivity to EGFR tyrosine kinase inhibitors is the first step of targeted lung cancer treatment. EGFR is a receptor tyrosine kinase normally found on surface of epithelial cells and is often overexpressed in a variety of human malignancies. The NCCN NSCLC Panel recommends testing for EGFR mutations and other biomarkers in patients with metastatic non-squamous NSCLC or NSCLC NOS, but also in squamous cell carcinoma [22,24,25].

The most common EGFR mutations include exon 19 deletions, present in 45% of the patients with EGFR mutations and p.L858R point mutations in exon 21, present in 40% of the patients with EGFR mutations, and are associated with responsiveness to oral EGFR TKIs [26,27]. Both mutations are found in approximately 10% of Caucasian patients with NSCLC and up to 50% of Asian patients [28]. Most patients with the common EGFR mutations are nonsmokers or former light smokers with adenocarcinoma histology [29], as well as younger age and female sex [30], although some studies show no statistically significant association between sex, histologic subtype and racial differences and EGFR mutation status [31,32], as well as with age, performance status or smoking status [32]. Although the two mutations are regarded as similar in predicting the benefit of EGFR TKIs, two sudies suggest better EGFR TKI benefit in patients with exon 19 deletions than in patients with exon 21 L858R substitution, with 50% greater therapeutic effect for the first ones, as well as longer overall survival [22,33], or response to platinum-based chemotherapy was observed only in patients with exon 19 mutations (RR=46% versus 0%, p=0.02) [34]. Other EGFR exon 20 mutations are diverse group of on-frame duplications or insertions, such as insASV, insSVD and insNPH and occur in approximately 2% of NSCLC patients and 4-12% of patients with EGFR mutations [35–38]. These patients have variable low response rates (9-25%) to erlotinib, afatinib or gefitinib [35,39], depending on the specific EGFR exon 20 insertion mutation [35,40,41], although there are rare exceptions (e.g., p.A763_Y764insFQEA) [36,42,43]. First-line platimum-based chemotherapy (± immunotherapy) is also recommended option for patients with EGFR exon 20 mutations (e.g. carboplatin/[pemetrexed or paclitaxel]) [44,45]. In this study, the frequency of EGFR was in accordance with 7.46%, almost equally present in both sexes.Exon 19 deletions and L858R were the two most commonly observed alterations, with only one patient with rare exon 20 mutation (L816Q), one patient with T790M and one with concomitant exon 19 and 20 EGFR mutation.

BRAF mutations

V-Raf murine sarcoma viral oncogene homolog B (BRAF) is a serine/threonine kinase, part of the canonical MAP/ERK signaling pathway. Activating mutations in BRAF result in unregulated signaling through the MAP/ERK pathway. The BRAF point mutation with change in amino acid at position 600 (p.V600E) is associated with responsiveness to combined therapy with oral inhibitors of BRAF and MEK, such as dabrafenib and trametinib. Other BRAF mutations are also observed in NSCLC, at a rate approximately equal to p.V600E, but their impact on therapy selection is yet to be understood and thus specific targeted therapy is not available [3]. Here, only V600E was detected in 4 (0.66%) of the 603 patients.

KRAS mutations

KRAS is a G-protein with intrinsic GTPase activity and activating mutations result in unregulated signaling through the MAP/ERK pathway. Approximately 25% of the patients with adenocarcinoma in a North American population have KRAS mutations [26,31]. KRAS mutation prevalence is associated with cigarette smoking, unlike many other actionable mutations [46]. Substitutions on KRAS G12, G13 and Q61 attenuate GTP hydrolysis capacity, while A146T promotes KRAS GTP formation in the form of increased nucleotide exchange, thereby reducing this isoform's oncogenic capacity [47]. The presence of a KRAS mutation, most commonly on

codon 12 (KRAS G12C), are prognostic for poor survival and to reduced responsiveness to EGFR TKI therapy [26,31,48], but are not predictors of RR, PFS or overall survival in lung cancer patients. However, the KRAS mutant is less sensitive to chemotherapy than the wild-type KRAS [47]. KRAS mutations do not generally overlap with EGFR, ROS1, BRAF and ALK genetic variants [49] and therefore, KRAS testing may identify patients that may not benefit from further molecular biomarker testing [3].

In this study, various KRAS mutations were detected in 50 patients (8.29%), approximately 90% of them on codon 12, which is in accordance with previously published data [47]. KRAS G12C and G12D were most common mutations, accounting for 20% and 14% of total KRAS mutations. Other KRAS mutations include G12V (12%), Q61H (2%), Q61L (2%), Q6H2 (2%), G13C (2%) and c.346G>T (2%). Codon 12/13 mutations are marked when KRAS mutations are examined using Cobas® kits. We detected 5 cases of co-occuring KRAS mutations, G12D with G12C, G13D and G12C with codon 12/13 mutation. Interestingly, an extremely rare case of 4 co-occuring KRAS mutations, G12D, G13D, Q61R and A146T were detected in 60 years old man with stage IVB adenocarcinoma (T4, N3, M1c).

PIK3CA mutations

PIK3CA encodes the lipid kinase PIK3, responsible for coordinating a diverse range of cell functions, including proliferation, cell survival, degranulation, vesicular trafficking and cell migration. Also, PIK3 is part of the PIK3/AKT signaling pathway important in oncogenesis and progression of lung cancer [50]. PIK3CA mutations are rare in NSCLC (1-4%); most of them are missense mutations in exon 9 (c.1624G>A (p.E542K), c.1633G>A (p.E545K)) and exon 20 (c.3140A (H1047R), M1043I, G1049S) that encode part of helical and kinase domains.The relation between PIK3CA mutations and age, sex, smoking status, histology and lymph node metastasis are unclear up to now [51]. PIK3CA mutations are correlated with both favorable and unfavorable prognosis of NSCLC patients, as it correlates with worse OS, shorter TTP, especially in patients treated with EGFR TKIs [48], poor PFS and Cancer-Specific Survival (CSS) [51], as well as with lymph node metastasis status [51]. Furthermore, they are probably not associated with primary resistance to EGFR TKIs in lung cancer patients. Moreover, acquired PIK3CA mutations related to EGFR TKI treatment are very rare [50].

In our retrospective cohort, PIK3CA mutations were observed in 1.82%; the frequency is consistent with previous studies [48]. Of them, 8 are point mutations on exon 9 (E542K and E545K) and 2-point mutations in exon 20 (H1047K). PIK3CA mutations were not mutually exclusive with KRAS mutation (G12C alone or with G12D+E542K, E545K and H1047R in three different patients), as it was for EGFR (one patient with H1047R PIK3CA+L858R EGFR), opposite to previous reports [48].

Co-occurring genetic alterations

The compendium of co-occurring genomic alterations in NSCLC are potentially more impactful than distinct mutations in oncogenic drivers with regard to determining tumor heterogeneity. In fact, NSCLC adenocarcinomas and squamous cell carcinomas have average number of somatic mutations per Mb that is higher than many other cancers [52].

In this cohort study, most oncogenic driver mutations occurred in a mutually exclusive fashion. Despite this, we identified 6 patients (1.00%) with two putative driver alterations. Most common were the KRAS/PIK3CA mutations, along with two patients with KRAS/BRAF mutations and one patient with EGFR/PIK3CA mutaiton. All of the identified co-occuring alterations in our study are well established based on data from previous studies, for example, the co-occurrence of PIK3CA and EGFR and KRAS in lung adenocarcinoma [49]. EGFR and KRAS mutations are known to be mutually exclusive [48], along with mutations in other established drivers, including ERBB2, BRAF, as well as ALK, ROS1 and RET rearrangements that are largely non-overlapping with KRAS [52]; this was also the case in our study. The clinical signficance of most of the co-occuring alterations at the time of primary diagnosis remains to be investigated, although for some co-occurring alterations is already well established. Such is, for example, the co-occurrence of EGFR and PIK3CA mutations, which is observed in 9.0-12.4% of advanced NSCLC adenocarcinomas [52]. In vitro, co-occurring EGFR and PIK3CA mutations promote cellular invasion and migration, while in vivo are associated with worse overall survival in some studies but do not impact RR and PFS with first- or second-line EGFR TKI therapy [53].

Unofrtunately, in our study, analysis of genetic alterations by specific histological subtypes was not relevant due to the significantly lower number of patients with squamous cell NSCLC than adenocarcinoma (4.48% vs. 92.87%) and the lower number number of squamous-cell cancer patients with genetic mutation (2.82% of all patients) than with adenocarcinoma (32.84% of all patients), not considering co-occurring mutations, in which no patient had squamous cell NSCLC. However, it is well estabilised that EGFR and KRAS mutations and EML4-ALK fusions are the most frequent driver alterations in lung adenocarcinoma, occurring with mutual exclusivity in 35-40% of tumors, along with HER2 and MAP2K1/MEK1 which are mutually exclusive of PIK3CA, BRAF, EGFR and KRAS mutations. In squamous-cell NSCLC, amplification of SOX2, PIK3CA, PDGFRA, FGFR1 and mutation of DDR2, AKT1 and NRF2 are most prominent, while TP53, BRAF, PIK3CA and MET mutations are common to both subtypes [54].

There are several potential limitations of this study: (1) the genetic alterations that might have been missed if they occurred outside the hotspot regions covered by the specific assays; (2) tumor biopsy specimens carry the inherent limitation that they do not capture intermetastatic tumor heterogeneity. Although driver mutations are considered as truncal events present at all sites of disease, other co- occurring genetic alterations could have evolved later and been present at sites other than the one at which biopsy was performed. Liquid biopsies (i.e., circulating tumor DNA analysis) and deeper sequencing technologies could help overcome these limitations; (3) the use of retrospective database that limits the ability to investigate other sources of potential bias; (4) the diagnostic and prognostic importance of the detected mutations could not be evaluated due to the lack of follow-up and (5) all patients were Caucasian, therefore the conclusions of the present study might not be generalizable to other racial and ethnic groups. An additional issue to be considered is the referral bias to provider enrollment of patients who were thought more likely to harbor targetable oncogenic mutation based on clinical and demographic features. This issue is reflected in the unusually high frequency of men vs. women (70.31% vs. 29.68%) included in the

study. Nevertheless, our genotyping results are consisted with the documented mutational prevalence of the tested oncogenes.

Conclusion

To sum up, our experience implementing systematic prospective genotyping for somatic alterations in EGFR, BRAF, KRAS and PIK3CA demonstrates the feasibility of this approach within the clinical workflow. The gained information supported diagnostic decisions and directed administration of available targeted therapeutics. As targeted therapy and immunotherapy have prompted a paradigm shift in advanced NSCLC treatment, demonstrating superior efficacy to chemotherapy alone both in second- and first-line setting [17], the next step in precision diagnosis and treatment of lung cancer will be the identification of novel molecular markers; the list of treatable oncogenic alterations and prognostic biomarkers is expected to signficantly expand [55].

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