Precision medicine in age-specific non-small-cell-lung-cancer patients: Integrating biomolecular results into clinical practice-A new approach to improve personalized translational research.

Precision medicine in age-specific Non-Small-Cell-Lung-Cancer patients: integrating biomolecular results into clinical practice - a new approach to improve personalized translational research.

Authors: Tiziana Vavalà & Valentina Monica*, Marco Lo Iacono, Teresa Mele, Simone Busso, Luisella Righi, Mauro Papotti, Giorgio Vittorio Scagliotti, Silvia Novello.

Department of Oncology, University of Turin AOU San Luigi, Regione Gonzole 10, 10043 Orbassano (TO), Italy;

***T.V. and V.M. are co- first authors.**

Corresponding author:

Silvia Novello, MD, PhD

University of Torino - Department of Oncology

San Luigi Hospital, Regione Gonzole 10, 10043 Orbassano (Torino), Italy

silvia.novello@unito.it

Phone : +39.011.9026978/233

Fax : +39.011.9038.616

Abstract

Objectives. Non-small-cell-lung-cancer (NSCLC) in young adults (≤ 45 years-old) accounts for a very small proportion, as this disease usually occurs in people at older age. The youthful NSCLC may constitute an entity with different clinical-pathologic characteristics, having predominance of adenocarcinoma histology and affecting mostly non-smoker subjects. However, without specific guidelines, it is currently considered, both clinically and biologically, as the same disease of the older counterpart, although differences have been documented.

Materials and Methods. Using formalin-fixed paraffin embedded diagnostic tissues (FFPE), targeted next-generation sequencing (NGS) technology allowed to provide insight the mutational pattern of 46 oncogenes and tumor-suppressor genes in 26 young patients (Y). Two additional populations, including a FFPE series of aged counterpart (A: 29 patients) and a group of healthy young controls (C: 21, blood provided), were also investigated to compare NGS profiles.

Results. Clinical features of enrolled young patients harmonized with literature data, being most of patients women (58%), never-smokers (38%) and with adenocarcinoma histology (96%)**.** C group was adopted to filter all the non-synonymous genetic variations (NS-GVs) not-associated with malignant overt disease. This skimmed selection mostly highlighted three genes: TP53, EGFR and KRAS. TP53 NS-GVs were numerically more numerous in younger, many involving specific annotated hotspot (R248, R273, G245, R249 and R282); the majority of EGFR NS-GVs was detected in young patients, with higher allelic frequency and mostly represented by exon 19 deletions. On the contrary, KRAS NS-GVs were mainly detected in aged population, with a prevalent compact pattern involving p.G12 position and associated with adenocarcinoma histology.

Conclusion. This retrospective study confirmed the feasibility of NGS approach for genetic characterization of NSCLC young adult patients, supporting the involvement of TP53, EGFR, and KRAS alterations in the early onset of NSCLC. Some of these GVs, or their pattern, may potentially contribute to customized targeted therapies.

Keywords

Youthful NSCLC, youngs, young patients, young adults, NGS, genetic profile.

1. Introduction

Lung cancer is the leading cause of cancer death in the developed countries [1]. Incidence is highest between the ages of 60 and 70, however the disease is diagnosed also among young patients, where has been found to have a rate from 1.2% to 6.2% (in patients under 40 years), 5.3% (in those under than 45 years) and 13.4% (in patients under 50 years) [2-6].

With the development of molecular targeted agents, 15% to 20% of non-small-cell-lung cancer (NSCLC) patients benefit from personalized approach based on the genetic background of the tumor [7]. Well-identified driver mutations in NSCLC include EGFR mutations, ALK rearrangements with already approved drugs available, but also other alterations in TP53, KRAS, LKB1, HER2, BRAF, MEK, ERK and AKT1, as well as RET fusions [8-12].

Despite the perception that molecular features are different in young adults with lung cancer compared to the overall population, this has not been clearly investigated: data are not always consistent among all series and age limits ranging from 40 to 50 years have been variably chosen by authors to define younger cohorts of patients [13-16].

The presence of a driver mutation in this particular population of patients reminds that lung cancer has an important heritable component, as evidenced by Hung et al., which identified several nicotine acetylcholine related genes in the 15q25 locus strongly associated with lung cancer [17]. Moreover, as evidenced by Sacher and colleagues, patients younger than 50 years are significantly more likely to have, than older patients, a driver mutation for which there is a growing array of evidence-based targeted therapies. In the absence of an identifiable treatable mutation, the youngest patients in the cohort studied also have a comparably unfavorable survival, suggesting an increased aggressiveness of the disease in young patients [18].

Understanding the etiology and biology of NSCLC in young adults compared with specific subgroups, as presented in this retrospective trial, will affect the management both from a public health standpoint of cancer control and by adjustment of therapy formulated for an older patient whose tumor may have a different biology.

2. PATIENTS AND METHODS

2.1 Study design, patients and biological samples

In this monocentric study, two age-specific cohorts of NSCLC cancer patients were identified and retrospectively collected at S. Luigi Gonzaga Hospital (Orbassano, Torino) between January 2007 and August 2014, together with a control population, in which the evaluation was done on peripheral blood.

Main inclusion criteria for patients were: age < 45 or > 65 years-old, histological or cytological confirmed lung cancer diagnosis according to WHO classification [19], enough FFPE (formalin fixed and paraffin embedded) histological or cytological specimens obtained by either surgical or needle biopsy/aspiration procedures, to perform molecular tests. Main inclusion criteria for control population were: age < 45 years-old, healthy voluntary adhesion to the study for which a written informed consent was signed to obtain a blood sample for molecular investigation. All tissue samples were de-identified and analyzed anonymously. For each patient and control, baseline information was collected about age, gender, ECOG performance status (PS), cancer familiarity and smoking habit. Never smokers, limited to tobacco habits, were defined as patients who had never smoked or smoked fewer than 100 cigarettes in their lifetime; former smokers were defined as patients who had a smoking history of at least 100 cigarettes in their lifetime and who, at

the time of the enrolment, were not smoking anymore since at least six months; current smokers were those who reported smoking at least 100 cigarettes in their lifetime and who, at the time of survey, smoked either every day or sometimes.

Tumor-related features were evaluated in terms of stage at diagnosis, histology. Moreover, EGFR mutations or EML4-ALK translocations were evaluated through routinely diagnostic procedures and each patient was categorized according to their molecular profile. Blood samples from control population were collected and molecular aspects compared with genetic variations' profiles obtained from the tissues in the two cohorts of patients. The mutational pattern for both tissue and blood samples was identified using next generation sequencing (NGS) approach.

2.2 Genomic DNA extraction and Next Generation Sequencing

After tissue manual microdissection, with enrichment for at least 50% of neoplastic cells, genomic DNA (gDNA) was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. gDNA was quantified using fluorometer Qubit platform (Thermo Fisher Scientific). NGS analyses were performed on the Ion Torrent Personal Genome Machine (PGM, Life Technologies, Grand Island, USA). Tumor samples were tested using a commercially available library kit (Ion AmpliSeq Cancer Panel, CP) to investigate 46 cancer-associated genes. Each amplicon library was generated starting from 10 ng of gDNA, as indicated by the manufacturer, and barcoded with Ion Xpress Barcode Adaptors Kit (Life Technologies). DNA Library quantification was performed using the PCR quantification kit and the 7900HT real time PCR system (Life Technologies), diluted in nuclease-free water to obtain a final concentration of 100 pM. Emulsion PCR was performed on Ion PGM™ Template One Touch 2 system (Life Technologies). The quality of

the emulsion PCRs was measured using the Qubit IonSphere Quality control kit (Life Technologies). IonSphere Particles with DNA were isolated and sequenced on Ion 316 Chip using the Ion PGM™ Sequencing 200 Kit (Life Technologies). Only sample sequences with at least a quality score of AQ20 (1 misaligned base per 100 bases) were considered for further analyses. The inclusion criteria for the analyses mainly considered the coverage target for each sample and this value was set at a minimum average deep of 100 reads for each amplicon (whole coverage: min=1, max=35388, amplicons median value=1801, amplicons average=1778). Variant Caller plugin included in Torrent Suite Software v.4.2.1 was used to identify variations in target regions and genetic annotation was performed with Annovar software [20](COSMIC database v.70, SNPs database v.138). Each of the identified genetic variation was coded according to "plus strand" of Human Genome assembly hg19.

2.3 Statistical analysis

Statistical correlation between gene variations with allelic frequency (AF) ≥10% and clinical-pathological features were investigated by Fisher exact test. Statistical analysis was elaborated using R statistical software[21].

3. RESULTS

3.1. Patients characteristics

Between January 2007 and August 2014, 26 patients, the NSCLC young adults group (Y), diagnosed in a range of age between 25-41 years-old, and 29 NSCLC patients > 65 age at the time of diagnosis cohort, defined as aged group (A), were retrospectively identified

together with a control population (C) of 21 young healthy volunteers. Patients' characteristics are shown in Table 1 and Table 2.

Y group was characterized by: median age 37 years; 15 females and 11 males; 20 advanced stage and 6 early stage; 16 smokers (10 current, 6 former) and 10 non-smokers; 25 adenocarcinoma/ 1 not otherwise specified (NOS).

A group was characterized by: median age 73 years (range: 68-82), 7 females and 22 males, 26 advanced stage and 3 early stage, 24 smokers (7 current, and 17 former) and 5 non-smokers, 16 adenocarcinoma/10 squamous cell carcinoma/3 other histology (1 pleomorfic carcinoma/2 NOS).

C subjects were characterized by: median age of 33 years (range: 26-45), 17 females and 4 males, including 16 never smokers and 5 smokers. Data about familiarity were not available for all the subjects and considering the purpose of the study, this issue was not planned to be discussed.

3.2 Cancer Panel genetic profile

DNA was successfully extracted, amplified and sequenced from all samples. Overall genetic variations (GVs) with ≥10% AF are reported in figure 1 and complete information for all genes included in analysis are presented in Supplementary Files [Table A.1 and Figures]. The pie charts on the left panel [Figure 1] summarize the variations pattern among the three groups: as expected, the C group showed a majority of synonymous (72.5%) and intronic (6.3%) alterations and, among non-synonymous (21.2%), the 90% included single nucleotide polymorphisms (SNPs), with no deletion/insertion detection; in the A dataset

the variations were subdivided in synonymous (55.4%), intronic (8%) and non-synonymous (36.6%), the latter including single nucleotide variations (SNV, 87%) and 13% of deletions/insertions/stop gain (Ins-Del-StopGain); in Y the variations detected were distributed among synonymous (53.6%), intronic (5.7%) and non-synonymous (40.7%), the latter including 79.4% SNVs and 20.6% of Ins-Del-StopGain. The middle panel contains all the variations affecting-protein sequence of each gene, in all subjects analyzed.

Being the C group characterized by subjects of age comparable with young adults patients and without malignant overt diseases at the time of the study (healthy controls), non-synonymous variations identified in control cases were removed from the analysis, in order to generate a graph including, hypothetically, only "tumor-related" GVs (right panel). Applying this filter, several genes previously found in the top ten rank, such as KDR, KIT and PI3KCA, were downgraded, because many GVs identified were shared by all groups, most likely as tolerated polymorphisms. The most frequent filtered non-synonymous GVs were mainly included in TP53 (#11 in 9 A, #17 in 13 Y), EGFR (#4 in 4 A, #12 in 8 Y) and KRAS (#6 in 6 A, #4 in 4 Y). The heatmap in figure 2 summarized this pattern distribution in patients.

3.3 Top three rate genes: TP53, EGFR, KRAS.

3.3.1 TP53

None of the TP53 variations identified was shared among Y and A patients, whereas the robust allelic frequency distribution in both group of patients suggests the high percentage of TP53 altered cells in both these tissues [Figure 2, upper section]. Furthermore, out of 12 patients who annotated familiar predisposition to cancer, 8 showed TP53 non-synonymous variations ($p=0.037$), targeting TP53.4 amplicon which covers a region of exon 2 ($p=0.02$). This data confirmed a fundamental involvement of TP53 in cancer onset. Also the smoking

habit seems to play a role in this context for Y patients: among 10 non-smokers, only two showed TP53 alterations, while in 16 smokers they were detected in 11 patients (p= 0.04), including 8 current and 3 former.

3.3.2 EGFR

As reported in the right graph of figure 1, the majority of EGFR variations was detected in Y: among the 12 variations (univocally considered), three were specifically found in A, 8 in Y and only one variation, the most known exon 19 deletion p.E746_A750del, was identified in 4 Y and 1 A, respectively. One additional patient was included for statistical elaboration, because of an alternative exon 19 EGFR deletion, p.L747_S752del. All EGFR exon 19 deletions identified were significantly correlated with female gender (p=0.035). In both patients groups, EGFR mutations were enriched in limited gene regions corresponding to the tyrosine-kinase domain, although in young adults' cohort some variations were also detected in the ligand-binding domain (figure mutation mapper [22]). To highlight, the two patients populations, as reported in the box plot of figure 2, showed interesting differences in EGFR mutations allelic frequencies, being significantly higher in young adults (median AF in Y= 39% ; median AF in A= 13%, t-test $p<0.01$). The heatmap included in figure 2 underlines both the increased amount of EGFR mutations in young patients and, by means of spots color intensity, their most consistent allelic frequency, all compared with aged counterpart. Those EGFR mutations with a clinical relevance for therapeutic choice were routinely tested by pyrosequencing, then confirmed by NGS.

3.3.3 KRAS

Opposite with EGFR GVs distribution, KRAS non-synonymous variations are mainly detected in A: the KRAS mutation p.Q131H was found in one out of 6 patients, while 5 out

of 6 patients (83%) showed a compact pattern involving the position p.G12 (p.G12C #3, p.G12S #1, p.G12V #1). Only two KRAS p.G12 substitutions (p.G12A #1, p.G12D #1) were described in Y, and two additional KRAS alterations were scattered along the domain (p.Q22* and p.Q61H, respectively). Restricted to A cohort, all 6 patients with KRAS non-synonymous mutations developed adenocarcinoma histology (p=0.02). Furthermore, we also observed the simultaneous presence of KRAS together with EGFR (3 patients) or with TP53 (7 patients) or with STK11 (2 patients) mutation.

4. DISCUSSION AND CONCLUSIONS

Scanty data are available specifically related to young patients with lung cancer and their molecular alterations and controversial results are reported in this field, boosting the need of specific guidelines for therapeutic action.

In this retrospective study, we collected and analyzed two groups of NSCLC patients, young adults and aged cohorts, and a series of healthy young controls. To the best of our knowledge this is the first study which aims to compare NGS genetic variations' profiles among a young patient population, the more common oldest and an additional healthy cohort; the control's samples were extracted from blood, in contrast with the samples generated by FFPE for Y and A populations. Respect to blood derived DNA, FFPE generated a DNA more degraded and with some artifact that could increase mutational rate. Disproportionate levels of C>T/G>A variations are often displayed in formalin-fixed samples in the 1-10% and less apparently in the 10-25% allele frequency range [23]. On this basis, although we used the range with minor artifacts, we could not compare directly the control population with the other ones, but we used it like a filter to minimize the effect of

annotated non-synonymous variations not associated, at the time of the study, with tumor evidence.

Clinical characteristics of enrolled young patients confirmed previous published data: most of patients were women (Y=58% vs A=24%), never smokers (Y=38% vs A=17%) and with adenocarcinoma histology (Y=96% vs A=55%). The reason for the high percentage of adenocarcinoma in younger patients is unclear; Subramanian et al. reported that NSCLC patients with no history of smoking appear to develop ~~lung~~ cancer at earlier ages compared with those who have a history of smoking [4]. However it is important to underline, as previously specified, that in our trial "never smoker" definition was considered from cigarettes only; this could be a limit of this study: the retrospective approach did not allow us to resume data about pipes, cigars, shisha or other equivalent, if present, above all in the Y cohort. Genetic factors have been suggested to play important roles in young patients with lung cancer: in a case control study, Schwartz et al, demonstrated the greatest contribution to lung cancer risk (7-fold increase) among 40- to 59-year-old non-smoker subjects, with a first-degree relative affected by lung cancer [24]. As compared with older patients, a higher female percentage in young patients, as in other publications, was present in our trial [5]. In literature there are also papers indicating the male as predominant gender [8, 13] and this might be related to different ethnicities and environmental effects [20]. Unfortunately, considering these aspects, for the limited number of patient, we are not able to discuss about some other features, such as occupational/environmental exposures, urban/rural residence influence on lung cancer predisposition for our population. Although these limitations, some molecular peculiarities among specific population-profiles were observed. Control group was arbitrarily established, but it was a good functional tool to filter all the non-synonymous variations

identified in healthy subjects at AF>=10%, therefore hypothetically not related to early-cancer onset, pointing out those with a concrete relationship with the overt disease. The subsequent removal of the "*tolerated polymorphisms*" evidenced the role of a selected top-three rank of genes, including TP53, EGFR and KRAS.

Not shared TP53 non-synonymous GVs were identified between the two groups of patients, being numerically more numerous in younger, many belonging to the annotated hotspot mutations of TP53. These specific hotspots (R248, R273, G245, R249 and R282), mostly occurring the DNA-binding core domain and leading to loss of target gene transactivation, are frequently mutated in human tumors [25-27]. In lung cancer, the effects of tobacco exposure increase DNA mutational ratio, including TP53 mutations [27]: this event could explain the association identified in young patients, where TP53 GVs are mainly collected in smokers, compared to non-smoker patients, where TP53 predisposition to alterate could be further enforced by carcinogens-exposure. This inclination to genetic alteration is clearly explained looking at EGFR data in NSCLC: as previously reported for TP53, the majority of EGFR non-synonymous mutations is strongly represented in young patients but, unlike TP53 results, EGFR mutations are often detected at higher allelic frequency in younger compared to the aged counterpart. According with previous data [28], EGFR exon 19 deletion, including both p.E746_A750del and p.L747_S752del, was mainly identified in young patients (5 out of 8 patients, 62.5%), less in aged counterpart (1 out of 4 patients, 25%), being in younger more frequent compared to EGFR mutation L858R (12,5%). EGFR exon 19 microdeletions have been documented as one of the preferential EGFR somatic alterations in subjects with specific germline polymorphisms, suggesting that EGFR exon 19 deletions have a pathogenic process distinct from other mutations [29], with a possible early onset compared to other EGFR mutation-sites. The prevalence of EGFR

exon 19 deletion in young patients further suggest the hypothesis of relationship between youthful lung tumors and genetic predisposition. In general, EGFR mutational pattern in young population suggests its driver role in the early NSCLC onset, further supported by its increased alteration rate (in our study 31%) compared to the literature-annotated data about EGFR mutation in Caucasian NSCLC (11-18%) [30].

In agreement with our data, a higher frequency of EGFR mutation and EML4-ALK rearrangement (11.6%) (in our study reached 23%) were also confirmed, by Sacher et al. in NSCLC ≤50 years compared with patients of all ages [18]. Similar observations were also evidenced by Gitlitz et al: preliminary data of the first, still ongoing, prospective trial (68 enrolled, age $\leq$ 39) about clinical aspects and genomic alterations in young lung cancer patients, evidenced mutations in EGFR (26%) and ALK (44%) [31].

The superior sensitivity of NGS method, compared with routine pyrosequencing method, allowed us to identify EGFR mutations at allelic frequency < 15% in patients previously considered EGFR wild-type. Moreover, with NGS technology a specific KRAS mutation pattern was identified, that could be potentially applicable in the future as prognostic or predictive factor. To support this hypothesis, Skoulidis et al recently reported that co-presence of KRAS, TP53 and STK11 mutations are related to 3 different expression profiles with distinct prognostic impacts [32]. Even if the aim of this study is not pointing the issue on prognosis and therapeutic approaches, the high frequency of gene mutations indicates that the detection of these alterations in young patients is really important and will help to identify the appropriate targeted drug therapy.

In conclusion, this is one of the first retrospective studies testing the feasibility of NGS for genetic characterization of NSCLC young adult patients. Although the restricted number of patients and study design limits the statistical power of the analyses, reported results are

in agreement with data previously published and suggest a peculiar mutational profile in young adult patients. In addition, our data support the involvement of TP53, EGFR, and KRAS alterations in young NSCLC patients. Some of these GVs or their pattern may potentially contribute, in case of confirmed data in larger cohorts, to customized treatments with targeted therapies.

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Conflict of Interest Statement

Prof. Giorgio V. Scagliotti is consultant for Eli Lilly and received honoraria from Eli Lilly, Roche, Astrazeneca, Pfizer and Clovis Oncology.
Prof. Mauro Papotti received honoraria from Ely Lilly, Novartis, Pfizer, Clovis Oncology.
Prof. Silvia Novello declared a role as Speaker Bureau for Roche, Boehringer Ingelheim, Eli Lilly, AstraZeneca, MSD.
All other Authors have no conflict of interest to declare.

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Table 1. Demographics.

	Young n = 26 N(%)	Adults n = 29 N(%)	Total n = 55 N(%)
Age (Years)			
Median	37	73	--
Range	(25-41)	(68-82)	--
Sex		Males versus Females p = 0.014	
Males	11 (42.3)	22 (75.9)	33 (60.0)
Females	15 (57.7)	7 (24.1)	22 (40.0)
Smoking Habit		Smokers versus Never Smokers p = 0.13	
Current	10 (38.5)	7 (24.2)	17 (30.9)
Former	6 (23)	17 (58.6)	23 (41.8)
Never smokers	10 (38.5)	5 (17.2)	15 (27.3)
Cancer Familiarity		Yes versus No p = 0.73	
Yes	6 (23.1)	6 (20.7)	12 (22)
No	14 (53.8)	21 (72.4)	35 (63.6)
Unknown	6 (23.1)	2 (6.9)	8 (14.4)
ECOG Performance Status at diagnosis		0 versus > 0 p = 0.11	
0	24 (92.3)	23 (79.3)	47 (85.5)
1	1 (3.8)	6 (20.7)	7 (12.7)
2	1 (3.8)	0 (0.0)	1 (1.8)

		Young	Adults	Total
		n = 26	n = 29	n = 55
		N(%)	N(%)	N(%)
Stage at diagnosis		Early versus Advanced p=0.28		
Early Stage				
	IA	1 (3.8)	1 (3.4)	2 (3.6)
	IB	1 (3.8)	0 (0.0)	1 (1.8)
	IIA	1 (3.8)	0 (0.0)	1 (1.8)
	IIB	0 (0.0)	1 (3.4)	1 (1.8)
	IIIA	3 (11.6)	1 (3.4)	4 (7.4)
Advanced Stage				
	IIIB	1 (3.8)	1 (3.4)	2 (3.6)
	IV	19 (73.2)	25 (86.4)	44 (80.0)
Histology		Adenocarcinoma versus non adenocarcinoma p << 0.01		
	Adenocarcinoma	25 (96.2)	16 (55.2)	41 (74.5)
	NSCLC, Not Otherwise Specified	1 (3.8)	2 (6.9)	3 (5.4)
	Pleomorfic carcinoma	0 (0.0)	1 (3.4)	1 (1.8)
	Squamous Cell Carcinoma	0 (0.0)	10 (34.5)	10 (18.3)

Table 2. Disease characteristics.