Original Research

Prognosis of ALK-rearranged non-small-cell lung cancer patients carrying TP53 mutations

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A B S T R A C T

Non-small-cell lung cancer (NSCLC) is the primary cause of cancer-related death. Gene rearrangements involving the anaplastic lymphoma kinase (ALK) tyrosine kinase identify a clinical and molecular subset of NSCLC patients, who benefit from the monotherapy with ALK tyrosine kinase inhibitors. Nonetheless, responsiveness to TKIs and TP53 mutations are evaluated in relation to disease control rate (DCR), objective response rate (ORR), progression-free survival (PFS) and overall survival (OS). Results: In patients with available clinical and TP53 mutation information, we found that 13 patients (20.3%) were affected by TP53 mutations. Considered together, even though showing a trend, TP53 mutations were not associated with PFS and OS. Considering the different TP53 mutations by functionality in terms of disruptive and non-disruptive mutations, we observed that TP53 non-disruptive mutations were able to predict worse OS in the overall case series. Moreover, a worse PFS was seen in the subgroup of patients with TP53 non-disruptive mutation, in first-, second-, and third line of treatment. Our results show that mutations affecting TP53 gene, especially non-disruptive mutations, are able to affect prognosis of ALK-rearranged NSCLC patients.

Introduction

Non-small-cell lung cancer (NSCLC) is the most commonly diagnosed cancer and the major cause of cancer-related death worldwide [1]. The anaplastic lymphoma kinase (ALK) gene is located in chromosome 2p23.2, and encodes for a single-pass membrane tyrosine kinase receptor, member of the insulin receptor superfamily. Binding of the ligand leads to receptor dimerization, auto-phosphorylation and signal transduction through JAK-STAT, PI3KCA-AKT, mTOR and MAPK pathways, resulting in cellular responses such as cell growth and resistance to apoptosis [2]; ALK rearrangements constitutively activate protein tyrosine kinase domain, leading to transforming downstream pathways [3].

A small inversion involving the echinoderm microtubule-associated protein-like 4 (EML4) and ALK was firstly reported in NSCLC in 2007 [4], and even though to date more than 20 genes have been identified as

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ALK fusion partners, the most frequent genomic variants are represented by different potential breakpoints affecting the EML4 gene, while the majority of fusion breakpoints for ALK falls before exon 20 of the gene, preserving the entire kinase domain [5,6]. Depending on the fusion partner and the genomic variant, the tyrosine kinase domain of ALK is constitutively activated through mechanisms affecting gene expression (the promotor region of the partner gene induces a constitutive transcription of the ALK mRNA), subcellular localization (mediated by partner domains) and ligand-independent phosphorylation (mediated by functional domains of the partner, e.g. coiled-coil) [5].

ALK rearrangements are usually mutually exclusive with respect to other driver mutations (e.g. EGFR, ROS1, RET), and characterize a specific subtype of oncogene-addicted NSCLC; these gene fusions occur in up to 8% of patients, and are mainly associated with clinical features such as adenocarcinoma histology, never or light-smoking history and young age [7–9].

Initially designed as a c-Met-tyrosine kinase inhibitor (TKI), crizotinib received FDA approval for ALK positive NSCLC patients after strong clinical results of a phase I/II study, later established as a first-line therapy for this subset of patients [10–12]. Thereafter, several second generation TKIs demonstrated a benefit in progression-free survival (PFS) and overall survival (OS) in crizotinib ALK-pretreated patients [13–16], until the displacement of crizotinib as a first-line therapy by brigatinib and alectinib [17–18].

Since the discovery of the first therapeutic agents for ALK positive patients, acquired resistance mechanisms have been highlighted, mainly classified as on-target or off-target mechanisms. On-target resistance mechanisms include ALK secondary mutations affecting the kinase domain, or ALK gene amplification, while the off-target ones involve signaling pathways that bypass the ALK tumor dependency, with the activation of alternative pathways, as HER2, EGFR overexpression, c-MET amplification or phenotypic changes guided by epithelial-mesenchymal transition (EMT) of cancer cells [19].

Beyond the cellular and molecular mechanisms induced by TKIs, response and prognosis of patients is affected by several pathways, which the most important probably is represented by Tumor Protein 53 (TP53) gene mutations. Mutations affecting TP53 demonstrated to play a pivotal role in influencing response to TKIs and prognosis of oncogene-addicted NSCLC patients, and it has been reported that different TP53 mutations could confer different functions to p53 protein, in particular those affecting exons 5–8 of the gene coding region: in particular, categorizing TP53 mutations in disruptive and non-disruptive mutations, basing on differences of protein structure and function of protein alterations, showed to predict different cellular functions, and an association with patient clinical outcome [20,21]. Basing on these results, the rationale to investigate the role of TP53 mutations in oncogene-addicted NSCLC is an emerging field of investigation to identify new prognostic and predictive biomarkers for this malignancy.

We previously showed that TP53 mutations, especially those affecting the exon 8 of the gene, affect response to first-line TKIs and prognosis of two independent cohorts of EGFR-mutated NSCLC patients [22,23]. At this regard, a recent article highlighted that specific TP53 mutations are involved in primary and acquired resistance to EGFR-TKIs. In particular, it has been demonstrated that exon 8 R273H mutations are able to guide primary and acquired resistance to EGFR-TKIs inducing epithelial-mesenchymal transition (EMT) effectors in an EGFR L858R/T790M cell line model, while this effect was not observed in an EGFR exon 19 deletion and TP53 R248Q model [24]. Moreover, another study found that TP53 mutations arise during resistance to osimertinib in EGFR-mutated patients, suggesting a role in guiding molecular pathways for resistance to TKIs [25]. In this study, we evaluated the role of TP53 mutations in predicting response to therapy and prognosis of ALK-positive NSCLC patients treated with TKIs.

Materials and methods

Data from all consecutive ALK-positive advanced NSCLC patients from July 2003 to February 2018 treated at the Medical Oncology Units of the Romagna catchment area (Area Vasta Romagna, AVR) and at the S. Maria della Misericordia Hospital of Perugia, Italy, were retrospectively retrieved. Medical and radiographic records were reviewed to obtain demographic and clinical features of patients, including tumor histology, age, gender, smoking history and information about treatments received, responses and clinical follow-up. ALK rearrangements were routinely assessed at the Pathological Anatomy Units of the centers involved in the study, by immunohistochemistry (IHC), Fluorescent in-situ hybridization (FISH), or both. A total of 98 records were obtained for this study; of these, 76 had available clinical and follow-up information and were considered for TP53 mutation and statistical analyses. All patients provided a written informed consent, and the study was approved by the CEROM Ethical Committee (study code IRST-B087).

TP53 mutation analysis

TP53 mutation analyses were performed on the same formalin-fixed paraffin embedded (FFPE) samples used for ALK rearrangement diagnosis, using the NM_000546.6 as a reference sequence. A dedicated expert pathologist from each Center accurately selected a tumor containing at least 50% of tumor cells for DNA extraction.

Following macro-dissection, cells were lysed in 50 mmol/L KCI, 10 mmol/L Tris–HCl pH 8.0, 2.5 mmol/L MgCl2, and Tween-20 0.45% digestion buffer. Proteinase K at 1.25 mg/mL were added and incubated overnight at 56 °C. Proteinase K was inactivated at 95 °C for 10 min, samples were centrifuged twice to eliminate debris and DNA quantity and quality in the supernatant was evaluated by Nanodrop (Celbio). TP53 mutation status was determined for the exons 5–8 by PCR amplification and Direct Sequencing using 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy) or Next-Generation Sequencing (NGS) Ion S5 platform (Thermofisher Scientific, Monza, Italy). NGS libraries were manually prepared starting from 10 ng of genomic DNA, using the AmpliSeq™ Library kit 2.0 and Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 (Thermofisher Scientific, Monza, Italy). Template preparation and enrichment were performed on a Ion Chef™ system with the Ion PGM Hi-Q View Chef kit. Sequencing was performed on the Ion PGM System using the Ion 316™ Chip v2. (Thermofisher Scientific, Monza, Italy). Signal processing and base calling were carried with the default base-caller parameters of Torrent Suite. Variants with <30 calls were filtered out. NGS analysis was performed using Ion Reporter software (v5.10). Limit of detection (LOD) for single nucleotide variants (SNV), insertions/deletions and splice site mutations was >3% mutant allele frequency (MAF) with a minimum depth of 500x. Frequencies of each single mutant were recorded and amplicon reads were reviewed with the Integrative Genomics Viewer (IGV), allowing visual inspection of the coverage of the regions of interest. Alignment and variant calling were performed using human reference genome 19 (hg 19), TP53 mutations were qualitatively classified as disruptive and non-disruptive mutations, as previously described [22,23]. Any mutation resulting in a stop codon, missense mutations occurring outside the L2 or L3 protein loops, and in-frame deletions within the L2 or L3 loops were categorized as disruptive mutations. Non-disruptive mutations were identified as missense mutation and in-frame deletions occurring outside the L2 or L3 loops and missense mutations within the L2 or L3 loops resulting in a substitution of one amino-acidic residue with another of the same polarity/charge.

Response evaluation

Best clinical responses to treatment to TKIs were evaluated on the basis of interval CT scans using standard Response Evaluation Criteria in Solid Tumors criteria (RECIST) version 1.1. In particular, responses to
treatments were classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). Patients with both diagnostic and at least one repeated evaluation after ALK-TKI monotherapy were considered for the study. All centers involved in the study used the same criteria for response evaluation.

Statistical analyses

Data were summarized by mean ± standard deviation (SD) for continuous variables and through natural frequencies and percentages for categorical ones. The association between categorical variables was tested by the Pearson’s χ² test or Fisher exact test, when appropriate, whereas those between a continuous variable and a categorical one was tested by means of the Student t-test or analogous non-parametric Wilcoxon-Mann Whitney test, when appropriate. Treatment responses were reported as objective response rate (ORR) and disease control rate (DCR). Objective response rate (ORR) was calculated as the ratio between complete response (CR) and partial response (PR), and the total number of patients evaluable, while Disease Control Rate (DCR) was calculated as the ratio between CR, PR and stable disease (SD) and the total number of evaluable patients. The time-to-event endpoints examined were progression-free survival (PFS) and overall survival (OS). With regard to treatment response and PFS, separate analyses for each line of treatment were performed. PFS was defined as the time from start of treatment to disease progression or death for any cause, whichever occurred first. Patients who were alive and progression-free at December 31, 2018, the last follow-up update, were censored at that date. With regard to PFS, separate analyses for line of treatment were performed. With regard to OS, the analysis was done on all patients. OS was defined as the time from date of diagnosis of advanced cancer to death for any cause. Alive patients were censored at the date of the last follow-up update. PFS and OS functions were estimated using the Kaplan–Meier method, and the log-rank test was used to assess differences between groups. Median PFS and OS were reported as point estimates and 95% confidence intervals (CI) in round brackets. The Cox proportional hazards regression model was used to quantify the association between specific covariates and the time-to-event endpoints. Results are reported as hazard ratio (HR) and 95% CI in round brackets. As the main study objective was to investigate an association between the presence of TP53 mutation or the type of TP53 mutation and PFS or OS, potential confounders (demographic or clinical covariates) of such relationship were studied comparing non-adjusted HR and adjusted one, that is including in the model other covariates other than the one related to TP53. If the percent-age difference between the two estimates was greater than 10%, confounding was considered present. Overall and when not otherwise specified, a two-sided p-value (p) <0.05 was considered statistically significant. All statistical analyses were performed using STATA 15.0 software (College Station, TX, USA).

Results

Clinico-pathologic and molecular features of patients

Retrospective data on 98 ALK-translocated NSCLC patients were obtained through medical chart review. Of these, 22 patients had no information on the clinical outcomes as well as on the treatment received; for this reason, analyses focused on 76 patients. Patients characteristics, including methodology for ALK assessment and TP53 mutations are reported in Table 1.

Of patients with available information on smoking history, 28 patients were never smokers (47.5%), 22 were former smokers (37.3%) and 9 were currently smokers (15.3%). Patients were never smokers (47.5%), 22 were former smokers (37.3%) and 9 were currently smokers (15.9%). The smoking status was evaluated on 64 patients with tissue availability for molecular testing: of these, 13 (20.3%) were affected by mutations: 4 in exon 5 (30.8%), 5 in exon 6 (38.5%), 1 in exon 7 (7.7%) and 3 in exon 8 (23%), while 51 patients (79.7%) had wild-type TP53. Accordingly to our previous works [22, 23], we qualitatively classified TP53 mutation into disruptive and non-disruptive mutations, finding that 7 patients had a disruptive mutation (53.8%) and 6 had a non-disruptive mutation (46.2%) (Table S1).

In the present study, 21 patients received an ALK-TKI agent as a first-line treatment, 57 as a second-line, and 28 patients as a third-line treatment. The type of ALK-TKIs received in each line is reported in Table S2. The total does not add up to 76 as some patients were treated with an ALK-TKI agent in more than one line. To investigate the association between TP53 mutations and response to treatment as well as progression-free survival, separate analyses for each line of treatment were performed. That is, on 76 ALK-translocated patients treated in first-line, on 67 patients in second-line, and on 36 patients in third-line.

Impact of TP53 mutations on response to treatment and progression-free survival

Response to treatment was evaluated as objective response rate (ORR) and disease control rate (DCR). In any line of treatment, no statistically significant association was found between TP53 mutations and ORR or DCR (Table S3). TP53 mutations were not correlated with ORR or DCR neither if classified as disruptive and non-disruptive mutations (Table S4).

In first-, second- and third-line treatment, median PFS was 4.59 (95% CI: 0.95-NA), 4.14 (95% CI: 0.59-12.98) and 3.55 months (95% CI: 0.16-NA) for TP53-mutated patients, respectively, while it was equals to 7.59 (95% CI: 4.93-11.14), 8.74 (95% CI: 5.42-12.42) and 11.76 months (95% CI: 2.99-19.97) for wt TP53 patients, respectively (Figure S1). No statistically significant associations were found in any line of treatment (log-rank test p-value equal to 0.203, 0.321 and 0.501, respectively).

When considering the different TP53 mutations in terms of disruptive and non-disruptive mutations, patients with non-disruptive mutations showed a worse prognosis. In first-, second and third line treatment, median PFS was 1.41 (0.82-NA), 3.91 (0.72-NA) and 1.91 (0.16-NA) for TP53 non-disruptive mutations, and 5.72 (95% CI: 0.76-NA), 4.14 (0.59-NA) and 34.17 (5.55-NA) for patients with TP53 disruptive mutations, respectively (Fig. 1).

Table 1 shows the results from Cox regression. At univariate Cox
Fig. 1. Impact of disruptive and non-disruptive TP53 mutations on Progression-free survival (PFS) of patients in first- (A), second- (B) and third-line of treatment (C).
analyses, patients carrying a TP53 disruptive mutation compared to wt TP53 patients were associated with a shorter PFS, especially in first- and third-line of treatment (HR = 2.49, 95% CI: 0.96–6.43, \( p = 0.059 \); HR = 2.02, 95% CI: 0.78–5.21, \( p = 0.146 \); HR = 5.85, 95% CI: 1.53–22.38, \( p = 0.010 \) for first-, second-, and third-line, respectively). No confounding effect by demographic and clinical covariates was observed.

Impact of TP53 mutations on overall survival

Median OS was 57.03 months (26.38-NA). Considering the patients by TP53 mutations, median OS was 48.88 months (26.38-NA) for wt TP53 patients and 67.77 (53.32-NA) for wt TP53 patients (Figure S2); TP53 mutations were also analyzed in terms of disruptive and non-disruptive mutations, and we confirmed that non-disruptive mutations are able to negatively affect OS (Fig. 2). At univariate analysis, the hazard ratio for patients with TP53 non-disruptive mutations as compared to wild type patients was 4.49 (95% CI: 1.49–13.58, \( p = 0.006 \)), while it was 1.05 (95% CI: 0.25–4.53, \( p = 0.943 \)) for patients with TP53 disruptive mutations as compared to wild type patients. No confounding effect by demographic and clinical covariates was observed.

Discussion

Following our previous results in two independent cohorts of EGFR-mutated NSCLC patients, in this study we analyzed the effect of TP53 gene mutations on clinical outcomes of ALK-rearranged NSCLC patients. Our results show that TP53 non-disruptive mutations predict worse clinical outcome of patients [22,23]. TP53 mutations are the most frequent in human cancers, promoting survival and resistance to apoptosis of cancer cells, with association to worse prognosis of cancer patients and resistance to systemic therapies [26,27]. Moreover, TP53 germinal are associated to Li-Fraumeni syndrome, confirming its role as a master regulator in human cancer [28].

TP53 is the most frequently mutated gene also in NSCLC, with mutation rates up to 55%, a predominant clonal expression [29–31]. On the other hand, in ALK-rearranged NSCLC patients, TP53 mutation rates range between 26% and 33% [32].

The prognostic role of TP53 mutations have been widely investigated in NSCLC, and several data showed that mutations affecting this gene are associated with worse patients prognosis [30,33-35], also affecting responsiveness to TKIs in the subset of EGFR-mutated patients [36-37].

In recent years, several studies investigated the role of TP53 mutations in predicting prognosis and responsiveness to TKIs in the subset of ALK-rearranged NSCLC patients.

Strong evidences and a recent meta-analysis indicate that TP53 mutations are strong indicators of worse prognosis in ALK-rearranged NSCLC patients [32,38-40]; furthermore, longitudinal assessment highlighted that these gene mutations are able to guide patients prognosis, and that the acquisition of TP53 mutation addressed patients to a

Table 2

|                       | First line |          |          |          |
|-----------------------|------------|----------|----------|----------|
|                       | HR (95% CI)| p        |          |          |
| wt TP53               | 1          |          |          |          |
| TP53 Disruptive mutation | 0.96 (0.38–2.43) | 0.927 |          |          |
| TP53 Non-disruptive mutation | 2.49 (0.96–6.43) | 0.059 |          |          |

|                       | Second line |          |          |          |
|-----------------------|------------|----------|----------|----------|
|                       | HR (95% CI)| p        |          |          |
| wt TP53               | 1          |          |          |          |
| TP53 Disruptive mutation | 1.05 (0.36–3.07) | 0.926 |          |          |
| TP53 Non-disruptive mutation | 2.02 (0.78–5.21) | 0.146 |          |          |

|                       | Third-line |          |          |          |
|-----------------------|------------|----------|----------|----------|
|                       | HR (95% CI)| p        |          |          |
| wt TP53               | 1          |          |          |          |
| TP53 Disruptive mutation | 0.28 (0.04–2.08) | 0.212 |          |          |
| TP53 Non-disruptive mutation | 5.85 (1.53–22.38) | 0.010 |          |          |
worse prognosis and shorter PFS following TKI treatment [41]. Robust data by Kron and colleagues confirmed that ALK-rearranged and TP53 co-mutations are predictive of both shorter PFS to ALK-TKIs, and shorter OS [41]; same evidence arose from three independent studies carried out in Asian patients, demonstrating that TP53 mutations predict low responsiveness to alectinib and crizotinib, and worsen OS [42-45].

Taken together, these evidences clearly indicate as TP53 mutations could negatively influence prognosis of patients, and even responsiveness to alectinib and crizotinib, and worsen OS [41].

These are the first evidences that specific non-disruptive vs disruptive mutations were observed.

Conclusions

In this study, we found that mutations affecting TP53 gene, especially non-disruptive mutations, are able to affect prognosis of ALK-rearranged NSCLC patients.

Data availability statement

The datasets generated for this study can be found by the Corresponding Author upon reasonable request.

Funding

This research received no external funding.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee Comitato Etico della Romagna (C.E.ROM.), protocol code IRST-B087, date of approval 06 March 2019.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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Supplementary materials

Figure S1: Impact of TP53 mutations on Progression-free survival (PFS) of patients in first- (A), second- (B) and third-line of treatment (C). Figure S2: Overall survival (OS) of patients in relation to TP53 mutations. Table S1: TP53 mutations of the patients case series. Table S2: ALK-TKIs admistration across the different lines of treatment. Table S3: Objective response rate (ORR) and disease control rate (DCR) according to TP53 mutations. Table S4: Objective response rate (ORR) and disease control rate (DCR) according to disruptive and non-disruptive TP53 mutations.

CRediT authorship contribution statement

Matteo Canale: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. Elisabetta Petracchi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. Paolo Gravero: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. Marita Mariotti: Writing – review & editing, Investigation, Data curation. Gabriele Minuti: Writing – review & editing, Investigation, Data curation. Giulio Metro: Writing – review & editing, Investigation, Data curation. Giovanni Martinelli: Writing – review & editing, Project administration, Investigation. Angelo Delmonte: Writing – review & editing, Visualization, Supervision, Resources,
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