Review Article

Non-small cell lung cancer molecular characterization of advanced disease with focus on sex differences: a narrative review

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Contributions: (I) Conception and design: R Bruno, G Alì, G Fontanini; (II) Administrative support: None; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: AM Poma, G Alì; (V) Data analysis and interpretation: AM Poma, R Bruno; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Molecular characterization is the basis of precision medicine in advanced non-small cell lung cancer (NSCLC), especially in the adenocarcinoma subtype. Determination of the molecular status of several predictive biomarkers (*EGFR*, *ALK*, *ROS1*, *BRAF*, *RET*, *NTRK* and PD-L1) is mandatory to tailor the therapy. Nowadays it is extremely clear that the definition of oncogene addiction and the evaluation of single biomarkers are insufficient to fully understand tumor biology and behaviour. In this context, peculiar molecular features with an impact on prognosis and a response to therapy can be related to sex differences. Lung cancer in non-smokers has been reported to be more frequent in women than in men, with a higher rate of driver and targetable alterations. A different molecular picture between men and women can depend on a sex-biased susceptibility to the carcinogenic effects of tobacco exposure and hormonal status. Although lung cancer in women has peculiar biological and clinical features, further studies are needed to better define sex-related molecular aspects and to evaluate to what extent they can improve personalized treatments.

Keywords: Biomarkers; lung cancer; precision medicine; sex differences

Received: 30 November 2020; Accepted: 01 April 2021; Published: 30 June 2021.

doi: 10.21037/pcm-20-72

View this article at: http://dx.doi.org/10.21037/pcm-20-72

Introduction

Lung cancer is the leading cause of cancer related mortality worldwide (11% of all cancers) with a prevalence of advanced stage in up to 70% of cases (1,2). In particular, lung cancer shows the highest incidence and mortality in males, and ranks third in incidence and second in mortality among females (2).

There are two main histological types of lung cancer: small cell lung cancer (SCLC), accounting for 15% of cases, derives from cells with neuroendocrine characteristics and has a highly malignant behaviour, and the more common non-small cell lung cancer (NSCLC), accounting for 85% of cases (3,4). NSCLC is further divided into the following subtypes: adenocarcinoma (ADC), which is the most frequent (40%); squamous cell carcinoma (SCC) (25–30%); and large cell carcinoma (5–10%) (4).

NSCLC subtype discrimination drives molecular analysis in the field of precision medicine. Molecular characterization is crucial for advanced lung adenocarcinoma patients, who can benefit from targeted therapies in presence of actionable gene alterations. According to the national and international guidelines, all patients with advanced adenocarcinoma—regardless of clinical indicators such as smoking status, race or sex—should undergo molecular testing for at least epidermal growth factor receptor (*EGFR*), ALK receptor tyrosine kinase (*ALK*), ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*), ret proto-oncogene (*RET*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), and neurotrophic receptor tyrosine kinase

(*NTRK*), whose alterations are predictive of response to approved kinase inhibitors. Furthermore, programmed cell death ligand 1 (PD-L1) evaluation in both ADC and SCC is necessary to select patients for immunotherapy (5,6). Other biomarkers are currently under investigation and their analysis is recommended whenever possible (6,7). Despite the initial response to targeted therapies, the majority of patients develop resistance within 1 year; consequently, alongside the identification of targetable alterations, an accurate molecular description of resistance mechanisms can improve patient management, leading to other treatment regimens (8,9).

It has been demonstrated that the evaluation of co-occurring mutations and the study of tumor heterogeneity are crucial to better understand the tumor molecular landscape, which can differently affect the response to therapies (10). In this respect, peculiar molecular characteristics and a different incidence of driver alterations mainly related to tobacco exposure and hormone regulation have been reported between males and females in lung cancer. Although not fully understood and described, sex-related molecular features can have important consequences on prognosis and response to therapy (11-15).

The aim of this paper is to review the most important predictive biomarkers in advanced NSCLC as well as the current knowledge about molecular differences between men and women with a brief reference to our seven-year single centre experience in histological and molecular diagnosis of lung cancer.

We present the following article in accordance with the Narrative Review Reporting Checklist (available at: http://dx.doi.org/10.21037/pcm-20-72).

Targeted therapies

Targeted therapies in lung ADC rely on the use of kinase inhibitors able to target driver oncoproteins, whose signaling pathways promote tumor survival and proliferation. Targetable alterations mainly occur in genes encoding for the tyrosine kinase receptors such as *EGFR*, MET proto-oncogene, receptor tyrosine kinase (*MET*), erb-b2 receptor tyrosine kinase 2 (*HER2*), *ALK*, *ROS1*, *RET*, *NTRK*, and for cytoplasmatic proteins with kinase activity including KRAS proto-oncogene, GTPase (*KRAS*) and *BRAF* (6,16).

EGFR

EGFR is the main actionable target in advanced lung adenocarcinoma, with a mutation frequency of 15% and 40% in Caucasian and Asiatic patients, respectively (17,18). Patients with advanced lung adenocarcinoma harboring *EGFR* activating mutations can achieve greater benefit from treatment with tyrosine kinase inhibitors (TKIs) than with platinum standard chemotherapy (19). *EGFR* mutations responsible for the constitutive activation of the protein involve exons 18, 19, 20 and 21, encoding for the tyrosine kinase (TK) domain. About 90% of cases are in frame deletions within exon 19 or the missense mutation L858R in the exon 21 (19,20).

Five TKIs have been approved for the first-line treatment of *EGFR* mutant lung ADC: the first generation TKIs erlotinib and gefitinib, which reversibly bind to the TK domain (21-23); the second generation irreversible TKIs afatinib and dacomitinib, which also inhibit HER2 (24-27); the third generation irreversible and mutant-selective osimertinib, active against both the sensitive mutations and the main resistance mutation T790M in the exon 20 (28,29).

Almost all patients treated with EGFR TKIs develop resistance within 1 year. The most important resistance mechanism to first and second generation TKIs is the secondary *EGFR* mutation T790M, which increases affinity for ATP (30). The presence of T790M, detected both in tumor tissue and in tumor circulating cell-free DNA (ctDNA), makes patients eligible for second-line treatment with osimertinib (28). Other significant resistance mechanisms are *HER2* amplifications (more common for first generation TKIs), *MET* amplifications, histological transformation in SCLC, and mutations in *BRAF*, *KRAS* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) genes (31).

Resistance mechanisms to osimertinib are more heterogenous and differ between first and second line settings. After second-line osimertinib treatment, in 10–26% of cases a tertiary *EGFR* mutation is detected within exon 20, the C797S, with or without a co-occurring T790M. Whenever C797S co-occurs with T790M, the definition of the allelic conformation (cis or trans) can drive the following line of treatment. Indeed, the co-occurrence of these two resistance mutations on the same allele (cis) leads to a mutant protein that cannot be targeted by currently approved TKIs. Otherwise, a TKI combination therapy can be suggested (32). At progression time to second-line osimertinib, about 49% of cases can lose T790M and also *MET* amplification and histological transformation are frequently observed (9). After first-

line osimertinib treatment, T790M is not detected, *MET* amplification and histological transformation are among the main resistance mechanisms, and the frequency of *EGFR* secondary mutations, like C797S, is about 10–15% (9,33).

It has been reported that several resistance mechanisms can co-occur in the same tumor, thus exacerbating the tumor heterogeneity issue. Consequently, a multi-gene analysis of both tissue re-biopsy and ctDNA is advisable (9,34).

Apart from the most frequent targetable alterations, about 10–14% of *EGFR*-mutated lung cancers harbor uncommon mutations alone or co-occurring with the most common activating mutations (i.e., exon 19 deletions and L858R) (35). The main sensitive rare mutations are E709x (1.5%) and G719x (1.5–3%) in exon 18, S768I (0.6–1%) in exon 20 and L861Q (3%) in exon 21 (20); patients harboring these mutations can be treated with afatinib (36). On the other hand, exon 20 insertions, which have an incidence of 4–10% among *EGFR* mutations, are well-known *de novo* resistance alterations, except for the insertion A763_Y764insFQEA, which has shown sensitivity to EGFR TKIs (37). Exon 20 insertions, for a long time considered untargetable, are currently under investigation with allosteric compounds, some of which (e.g., poziotinib), have already shown promising results, with a reported response rate of 64% in a phase-II clinical trial (38,39).

BRAF

BRAF encodes for a serine threonine kinase and is mutated in 5–8% of NSCLC, with a higher prevalence in lung ADC (40,41).

In advanced lung ADC, the V600E mutation, accounting for 50% of all BRAF mutated cases, is a predictive biomarker (42) designed to select patients for front-line treatment with dabrafenib (a BRAF inhibitor), together with trametinib (a MEK inhibitor) (43). Resistance mechanisms to BRAF and MEK TKIs have not been clearly described in lung cancer, but the principal ones include MEK activating alterations, *RAS* mutations, phosphatase and tensin homolog (*PTEN*) inactivation, as well as tyrosine kinase receptors overexpression (44).

BRAF mutations have also been described as resistance mechanisms to some TKI treatments as those targeting EGFR or ROS1 (31,45).

Finally, it is worth noting that *BRAF* V600E mutation in lung cancer as well as targetable alterations in *EGFR*, *ROS1* and *ALK*, are associated with an immunosuppressive tumor microenvironment, whereas non V600E mutations (i.e., K601E, L597Q, G469A), more frequently detected in smokers, are linked to an immunogenic tumor microenvironment. This aspect can have implications in patient selection for immune check-point inhibitors (46).

MET-HER2-KRAS

MET alterations have been described in advanced lung ADC as both primary oncogenic drivers and drivers of acquired resistance to EGFR TKIs (47). *MET* amplification and alterations causing the skipping of exon 14 can be targeted. More specifically, *MET* amplification is reported in 2–5% of NSCLC (48), and its incidence is higher after treatment with EGFR TKI (9,31). Combination therapies including EGFR and MET inhibitors are currently under investigations (47). *MET* exon 14 skipping alterations are reported in 2–4% of NSCLC and are the most common alterations in sarcomatoid carcinomas (49,50). Interestingly, in 5% of cases *MET* mutations can co-occur with other driver alterations mainly in *RAS* genes (51). The multi-kinases inhibitors crizotinib and cabozantinib have been approved by FDA as a breakthrough-therapy designation for the treatment of patients who progressed after receiving platinum-based chemotherapy, and capmatinib was approved also for first-line setting (52). The MET-selective inhibitors capmatinib, savolitinib, and tepotinib also showed valuable results especially when used in combination with anti-EGFR TKIs (53,54). Tepotinib was approved by the FDA with a breakthrough-therapy designation of patients—affected by metastatic NSCLC harboring MET exon 14 skipping alterations—who progressed after platinum-based chemotherapy (55).

HER2 is a tyrosine kinase receptor from the same family of EGFR. Its main alterations in lung ADC are exon 20 insertions (2–9% of cases) (56) and amplifications, with the latter mainly described as EGFR TKI resistance mechanisms (57). The targeted drugs currently available have a limited activity in HER2-mutant NSCLC, but none of these have been approved so far (58). However, poziotinib has shown promising preclinical and early clinical activity in NSCLC patients with *HER2* or *EGFR* exon 20 insertions (39,59).

KRAS encodes for a GTPase protein and is mutated in 20–30% of lung ADC patients, mainly smokers, with G12C being the most frequent alteration (40%) (7). Over the years, several attempts have been made to target this key protein for tumor progression, but promising results have

been obtained only recently with the development of KRAS G12C specific inhibitors, among which AMG159 and MRTX849 are currently investigated (60).

Gene fusions

The *ALK* gene is rearranged in 3% to 5% of NSCLC cases (61), typically occurring in younger, in never- or light-smokers, and in adenocarcinoma patients (62). The most frequent *ALK* rearrangements are caused by pericentric inversions of the short arm of chromosome 2, leading to a fusion gene between the amino terminal portion of the Echinoderm Microtubule-associated protein Like 4 (*EML-4*) gene and the juxtamembrane region of the *ALK* gene (63). As well as *EML4*, more than 90 other *ALK* partners have been reported in NSCLC, including the kinesin family member 5B (*KIF5B*), trafficking from ER to golgi regulator (*TFG*), the kinesin light chain 1 (*KLC1*), and the huntingtin interacting protein 1 (*HIP1*) (64,65). The multi-kinases inhibitor crizotinib (first generation ALK TKI) is the first to have been approved to treat ALK-positive lung cancer patients (66,67). Despite its initial significant benefit, the efficacy of crizotinib decreases after the onset of acquired resistance mechanisms, mainly secondary mutations in the *ALK* gene and activation of bypass tracks (68). Several innovative ALK inhibitors have been approved in both crizotinib-naïve and resistant *ALK*-rearranged NSCLC including ceritinib, alectinib, brigatinib and lorlatinib. At present, alectinib is also the preferred agent for first-line treatment (69).

ROS1 is activated by chromosomal rearrangement in about 1% of NSCLC cases (70,71). Well-known *ROS1* fusion partners in lung cancer include the CD74 molecule (*CD74*), which is the most frequent, DEPP1 autophagy regulator (*DEPP1*), the solute carrier family 34 member 2 (*SLC34A2*), and syndecan 4 (*SDC4*). The kinase domains of *ALK* and *ROS1* share 77% of amino acid identity within the ATP-binding sites and can be targeted by the same inhibitors, including crizotinib (67). Ceritinib, lorlatinib (72,73), entrectinib (74), and repotrectinib have also proved to be safe and active in patients with advanced ROS1-rearranged NSCLC (75).

RET gain-of-function mutations or rearrangements occurs in 1–2% of NSCLC patients (76). *RET* rearrangements involve at least 12 fusion partners, the most common being the kinesin family 5B (*KIF5B*) gene, followed by the coiled-coil domain containing 6 (*CCDC6*) gene, the nuclear receptor coactivator 4 (*NCOA4*) gene or the tripartite motif containing 33 (*TRIM33*) gene (77). *RET* fusions are mutually exclusive with other driver mutations and more commonly appear in younger patients and light-/never-smokers (78). Selpercatinib has recently been approved for the first-line treatment of RET-rearranged NSCLC according to the results of the LIBRETTO-001 trial (79). In addition, cabozantinib proved to be effective as second-line treatment with a median progression free survival (PFS) equal to 5.5 months and a median overall survival (OS) of 9.9 months in 25 patients included in a phase II-trial (80).

The tropomyosin receptor kinase (TRK) family consists of three tyrosine kinase receptors: TRKA, TRKB and TRKC isoforms, encoded by the *NTRK1*, *NTRK2* and *NTRK3* genes, respectively. They are predominantly expressed in the nervous system, where they act as important modulators in the development of neuronal and other tissues (e.g., lung, bone) (81). *NTRK* fusions involve over 80 partner genes that promote constitutive TRK signaling activation, cell transformation, and proliferation (82,83). *NTRK* fusions have a prevalence of 0.1–1% in unselected NSCLC, which reaches about 3% in tumors lacking *EGFR*, *KRAS*, *ALK* and *ROS1* alterations (81,82). In the last two years, FDA has granted accelerated approval to the two first-generation selective TRK inhibitors, larotrectinib (84) and entrectinib as tumor-agnostic treatment after the impressive clinical activity in patients whose tumors harbored NTRK fusions (85). Future clinical research in the setting of these tumors is directed towards overcoming resistance to first-generation TRK inhibitors.

Immunotherapy

Immunotherapy aims to induce or enhance the cancer-specific immune response and, in this context, the use of check-point inhibitors has been approved as first- or second-line treatments in different solid tumors. PD-L1/PD-1 is the most important immune checkpoint, associated with immune tolerance and autoimmune diseases, and is used by the tumor to elude the immune system. PD-1 is encoded by the programmed cell death 1 (*PDCD1*) gene, and it is expressed in immune cells such as T-cells, B-cells, natural killer cells, and dendritic cells. PD-L1 was found to be not only confined to the surface of tumor cells, but also expressed on the surface of antigen-presenting cells (APC), Treg cells, infiltrating myeloid cells, and others. When binding to its ligands, PD-1 can inhibit the activation of lymphocytes and induce the death of lymphocytes (86,87).

Therefore, agents targeting the PD-L1/PD-1 axis have become a hotspot of anti-tumor treatments. The PD-L1 protein expression detected by immunohistochemistry (IHC) is the predictive biomarker used in most immuno-oncology clinical trials (88).

Anti PD-1 pembrolizumab and nivolumab and anti PD-L1 atezolizumab are approved by FDA as monotherapies for advanced NSCLC. Based on the outcomes of a series of clinical trials, PD-L1 testing has been recommended to select patients for Pembrolizumab monotherapy as first-line treatment (PD-L1 ≥50%) (89-92). The predictive value of PD-L1 expression has proven to be quite satisfactory for first-line pembrolizumab/atezolizumab monotherapy, but it is not ideal to select patients for pembrolizumab-based combination regimens as first-line treatments [Keynote-189 and Keynote-407, (93,94)]. In the second-line setting, Checkmate-017 and Checkmate-057 studies have demonstrated superior OS of nivolumab monotherapy over docetaxel, regardless of the PD-L1 expression (95,96). However, POPLAR and OAK have reported that only patients with a PD-L1 expression on tumor cells or on immune cells greater than 1% could benefit from immunotherapy, while patients with expression levels below 1% did not achieve a better OS compared to docetaxel (97,98). Variable PD-L1 predictive values have been reported across different clinical trials testing anti PD-1 or PD-L1 agents, and different studies have shown the imperfect predictive value of this biomarker.

In this respect, a better determination of tumor antigenicity and tumor microenvironment can help to identify patients eligible for immunotherapy. Tumor mutational burden (TMB) is related to the number of mutations per megabase. A high number of somatic mutations may lead to a greater number of neoantigens presented on the surface of tumor cells, which, in turn, may increase immunogenicity (99). The relation between a higher mutational burden and a better response to checkpoint inhibition has been demonstrated in several studies. In particular, the post-hoc analysis of Checkmate 227 demonstrated that a higher TMB is associated with prolonged PFS independent of PD-L1 expression and histology in patients receiving first-line nivolumab plus ipilimumab in a metastatic setting. However, OS revealed no benefit related to high TMB (100). In spite of its potentiality, TMB is not a biomarker easy to evaluate in clinical practice; in addition, a standardization procedure and a univocal cut-off are still missing.

Immune gene signatures have been also evaluated to characterize the tumor microenviroment. In particular IFN-γ signaling and activated T-cells have shown a good predictive value in identifying responders to immunotherapy across several solid tumors including melanoma and NSCLC (101).

Molecular tests

The increasing number of predictive biomarkers in lung cancer and the development of new drugs make it necessary to perform a complete evaluation of tumor molecular status. However, molecular characterization of advanced lung cancer can be hampered by the availability of biological material. As a matter of fact, most lung cancer patients are diagnosed at advanced stage, and in about 50% of cases only cytology is available (102).

Compared to other molecular tests, the use of next generation sequencing (NGS) multi-gene panels is recommended, since NGS panels allow to evaluate several markers simultaneously by using a few biological materials. NGS is the ideal method to analyse also rare mutations seldom included in hotspot tests, thus providing additional information that is relevant to the clinical context (35,103). Moreover, the use of NGS is necessary to explore tumor heterogeneity and to identify co-occurring mutations with an important impact on prognosis and response to therapies (10,104). NGS panels are also an advantage for the analysis of ctDNA, which is essential to monitor the response to treatment and to evaluate resistance mechanisms (105).

Different diagnostic algorithms regarding gene fusions are currently used, mainly based on fluorescent in situ hybridization (FISH) and IHC. The detection of the ALK fusion protein by IHC is the gold standard to select patients eligible for ALK TKI treatment. However, moderate or weak ALK immunostaining requires FISH confirmation (106). *ROS1* rearrangements can be detected by FISH assays using a dual-color break-apart probe, but the detection of elevated ROS1 protein levels by IHC may provide cost-effective screening, considering the rarity of these rearrangements in NSCLC. Confirmation of positive or doubtful ROS1 immunostaining by FISH or by other methods is highly recommended (106). FISH and sequencing techniques are considered the standard methods to detect *RET* rearrangements, considering the low accuracy demonstrated by IHC (76). NGS is the most commonly used and specific method to detect *NTRK* fusions (107). IHC can detect TRK proteins overexpression, which may reflect the presence of *NTRK* fusions. This approach has

shown an overall sensitivity of 88%, which is lower for *NTRK3* fusions (79%) than *NTRK1* and *NTRK2* (about 97%) (107). IHC can therefore be used in clinical practice as a screening method to identify *NTRK* fusions followed by a confirmatory NGS test. Despite the cost-effectiveness of FISH and IHC, the evidence that specific fusion variants can have a different sensitivity to TKIs (108) has favored the development and validation of several multi-marker panels running on both NGS and non-NGS platforms (109-111).

Although there is a lack of standardization and expertise for a correct interpretation of the results, multi-marker NGS panels are currently the main protagonists of lung cancer molecular characterization, with different panels already validated and approved for clinical practice (112).

Sex impact on the molecular landscape of NSCLC

Sex differences can impact on incidence, prognosis, mortality and response to therapies (14). To date, the molecular basis for sex disparities is still to be fully understood, but it has been demonstrated that men have a higher mortality rate than women, who better respond to both surgery and chemotherapy (113).

Sex differences are mainly due to smoking habits and hormonal status (114,115). Epidemiological studies have demonstrated that lung cancer in women is less associated with tobacco exposure. In fact, lung cancer in never smokers has an incidence of 10–15%, and is more frequent in women (53%) than in men (15%) (114). Among others, an increased risk of lung cancer in women can also be correlated to biomass fuel and cooking and to a higher susceptibility to chronic obstructive pulmonary disease than in men (114).

Discordant data have been reported concerning different risks of developing smoking-related lung cancer in males and females (114,116). However, some differences in the carcinogenic effects of tobacco have been identified: in women, cytochrome P450 family 1 subfamily A member 1 *(CYP1A1)* is highly expressed, and glutathione S transferase M1 *(GSTM1)* has a higher rate of mutations or polymorphisms that decrease its activity (117). CYP1A1 activates polycyclic aromatic hydrocarbons to highly reactive compounds capable of binding DNA, whereas GSTM1 detoxifies active forms of polycyclic aromatic hydrocarbons (117). Accordingly, female smokers have higher levels of DNA adducts compared to male smokers. Tumor protein p53 (*TP53*) mutations have a higher frequency in smoking women than in smoking men, whereas the opposite is observed in non-smokers (118). Finally, a lower efficiency of the DNA repair system has been revealed in both smoker and non-smoker women (119-121). Adenocarcinoma is the most frequent histotype of NSCLC, with a higher prevalence in women, who more often present with driver alterations in targetable oncogenes. *EGFR* mutations and *BRAF* V600E are more frequent in non-smoker females than in non-smoker men (114,122). Similarly, *HER2* mutations and *MET* exon 14 skipping mutations are associated with female sex, never-smoked status and adenocarcinoma histology (51,123). On the contrary, *KRAS* mutations are more common in smokers, with G12C being the most frequent alteration, especially in women (124). Comprehensive molecular profiling of lung ADC performed on tumor and paired normal tissues from 230 untreated lung ADC patients revealed that only a fraction of significantly mutated genes is enriched in men or women. Among these genes, *EGFR* mutations were more frequent in females, whereas loss of function mutations in the gene RNA binding motif protein 10 (*RBM10*), located on chromosome X, were more common in men (125). Other X-linked genes were confirmed to play an important role in the biology of lung cancer. These genes included the Gastrin-peptide growth factor receptor (*GRPR*), which is more commonly expressed in women than in men both smokers and non-smokers. This receptor enhances cell proliferation and metastases and can be regulated by estrogens (126).

Metabolic abnormalities in lung cancer differ between sexes, as demonstrated by Li and collaborators who performed a transcriptome analysis using The Cancer Genome Atlas (TCGA) data. In particular, they identified metabolic genes differently expressed and impacting on prognosis in a sex-biased manner. TAO kinase 2 (*TAOK2*), a serine/threonine protein kinase catalytically activated during mitosis, was found to influence male prognosis. On the other hand, N-acylsphingosine amidohydrolase 1 (*ASAH1*), which catalyzes the hydrolysis of ceramide into sphingosine and may act as an oncogene, seems to have a critical role in women. The authors identified a total of 34 and 15 metabolic genes in men and women respectively, as potential diagnostic and prognostic sex-biased biomarkers for lung ADC (127).

Hormonal status can explain some sex differences in human tumors considering that estrogens are crucial for cancer development (128). For instance, 17-b-estradiol impacts on lung cancer and can activate the EGFR

pathway (129). The estrogen receptors (ER) expressed in the lung epithelium are ER-alpha, which allows a proper differentiation of the lung and an adequate number of alveoli per surface, and ER-beta, which is involved in the development of the extracellular matrix (130). ER-beta is associated with *EGFR* mutations (131), and is highly expressed in lung cancer, especially in premenopausal women. Consistently, the inhibition of aromatase, crucial for estrogen synthesis, may improve the response to EGFR TKIs (129).

Cheng and collaborators examined the expression of the hormone receptor in lung tumors, finding that women had a lower cytoplasmatic ER-alpha and nuclear ER-beta expression than men. Higher cytoplasmatic ER-alpha and nuclear ER-beta expression is associated with a worse survival (132).

Dubois evaluated the contribution of lymphatic and blood endothelial cells in the sex-dependent modulation of lung cancer. They discovered that lung tumors had a faster growth in female than in male mice, and that estradiol specifically promoted tumor development in females. They observed that estradiol increased lymph-angiogenesis and the levels of the vascular endothelial growth factor A (VEGFA) and of the fibroblast growth factor 2 (bFGF) through the ER-alpha pathway. They also reported that the use of tamoxifen and of ER-alpha antagonist decreased lung tumor volume, altered blood and lymphatic vasculature, and reduced VEGFA and bFGF levels only in females (133).

Cancer in males and females also present some endocrine differences. Parathyroid hormone-related protein (PTHrP) is expressed in NSCLC and is upregulated in tumors presenting skeletal metastasis. It has been demonstrated that the PTHrP expression has a positive impact on OS in females, whereas survival in males is independent of PTHrP (134,135).

Sex differences were also reported in both innate and adaptive immune responses, with more antigenic tumors in men. Immune checkpoint inhibitors tend to be more effective in male patients, while immunotherapy combined with chemotherapy is more effective in females. These sex differences may again result from hormonal factors and X-linked genes (11,136). Immune-related adverse events are more frequent in women, especially pneumonitis and endocrinopaties, but a better PFS was observed in women with adverse events (136). Caetano et al demonstrated a sex-specific role for epithelial STAT3 signaling in the *KRAS*-mutant mouse model of lung adenocarcinoma. Specifically, the absence of epithelial STAT3 in males favours lung tumorigenesis through IL-6 signaling and neutrophilic inflammation, which is diminished by estrogen/ER signaling in females (137).

A recent meta-analysis has evaluated the impact of sex and age on the treatment of lung cancer by analysing data from representative Phase III-clinical trials and also by evaluating TKI efficacy. The authors reported sex differences related to treatment efficacy: women respond better whenever the drug shows activity, and this suggests that treatment should be evaluated also considering sex (138). Likewise, Buonerba and collaborators found that male smoker patients positive for L858R *EGFR* mutation may benefit less from EGFR TKI treatment (17).

Yuan *et al.* reported that in the lung adenocarcinoma cohort of TCGA, the serine/threonine kinase 11 (*STK11*) gene is more frequently mutated in males; and that *STK11* inactivating mutations may predict sensitivity to mTOR and SRC inhibitors (139). On the other hand, neurofibromin 1 (NF1) in SCC is more frequently deleted in women and its inactivation is associated with mTOR and MEK inhibitors sensitivity (139-141).

Finally, Radkiewicz and collaborators reported that sex differences are less evident in SCC than in ADC and they confirmed a different rate of *EGFR* mutations (15).

Sex differences in lung cancer: a single centre seven-year experience

The availability of large databases and NGS techniques has increased our knowledge of sex differences in lung cancer, but only a few data are available regarding differences in targetable alterations between Caucasian men and women with advanced lung cancer. In this paper, we report data from our single centre seven-year experience including 2,425 consecutive patients diagnosed with advanced lung cancer, who underwent molecular characterization at the Unit of Pathological Anatomy of the University Hospital of Pisa. In detail, 1,514 men (median age 68.2±10.7 years) and 911 women (median age 65.4±9.4 years) were tested for the mutational status of *EGFR*, *BRAF*, *KRAS*, *MET* and *PIK3CA*; in addition, *ALK*, *RET* and *ROS1* fusions, as well as *HER2* and *MET* amplifications were also evaluated. *Figure 1* shows the histological and molecular data.

The SCC histotype was significantly more frequent in men, while the *EGFR* mutations were more common in women. Similarly to *EGFR*, *PIK3CA* alterations tended to be more frequent in women. No differences were observed for the other biomarkers. Interestingly, exon 19 *EGFR*

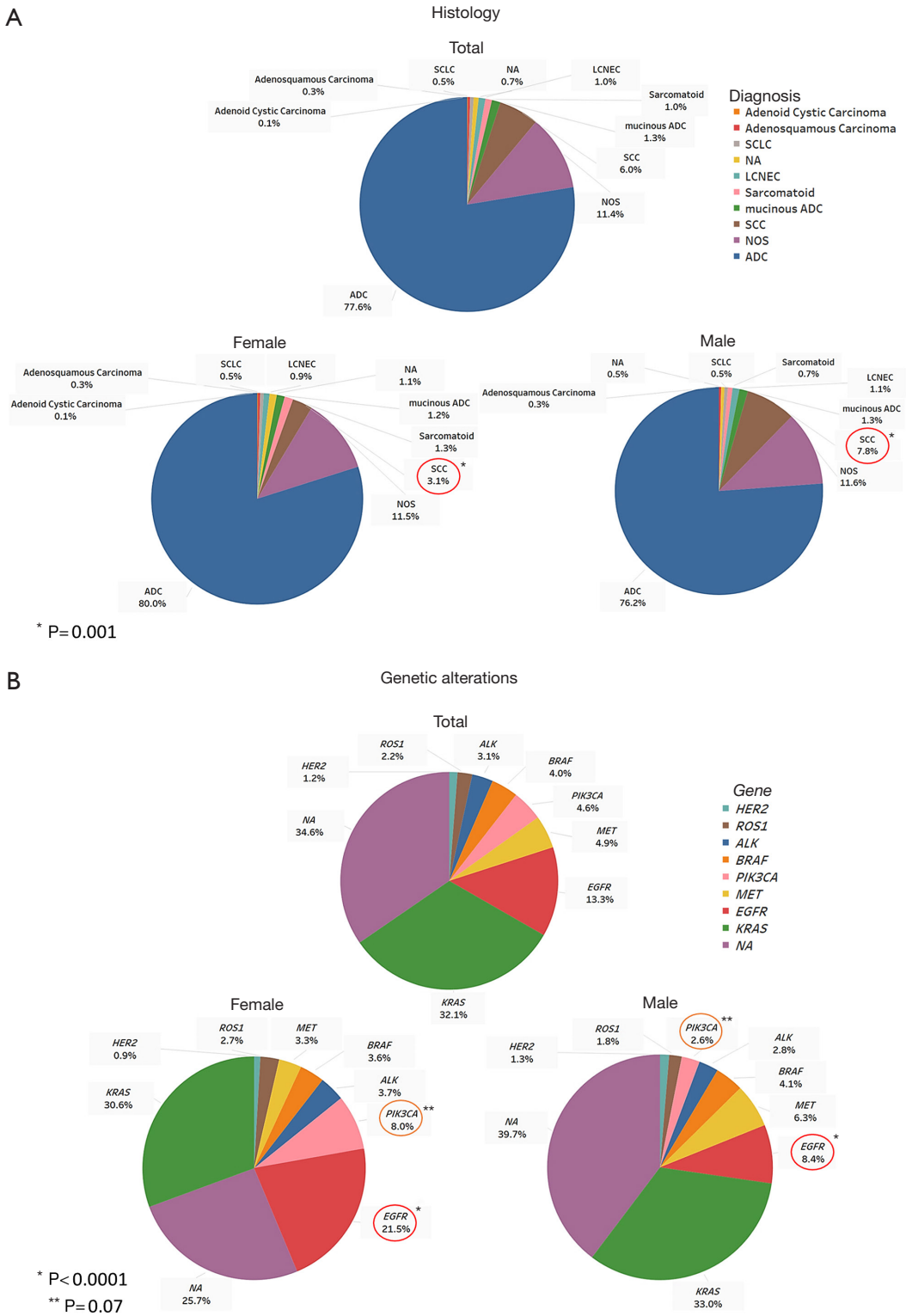

Figure 1 Histological diagnoses (A) and molecular status (B) in the whole cohort and by sex. Red circles indicate significant differences; trends are highlighted by orange circles. ADC, adenocarcinoma; SCLC, small-cell lung cancer; NA, not available; LCNEC, large cell neuroendocrine carcinoma; SCC, squamous cell carcinoma; NOS, not otherwise specified.

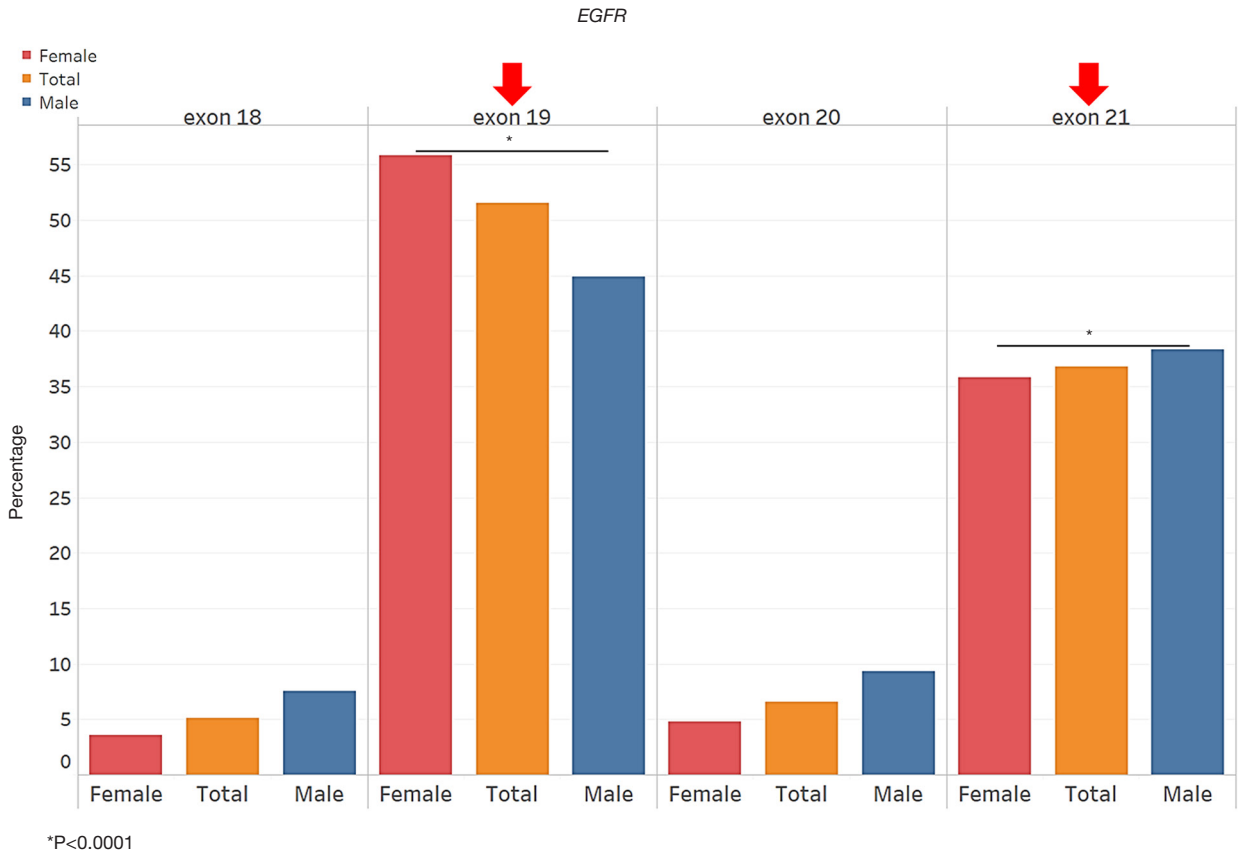

Figure 2 *EGFR* mutations distribution by sex. Red arrows indicate significant differences.

deletions were significantly more frequent in women, whereas L858R was more frequent in men (*Figure 2*).

The rate of *EGFR* mutation types associated with sex has not been clearly assessed, but it has been demonstrated that exon 19 deletions usually occur in younger patients (142), and, in our cohort, females were significantly younger than men (P<0.001). Koyama and collaborators found that patients with tumors harboring an exon 19 *EGFR* deletion have better OS compared to L858R after treatment with EGFR TKI (143), thus supporting the overall better response to TKIs in women. As regards the TKI resistance mechanisms, no differences were observed between tumors harboring exon 19 deletions and L858R (144). *PIK3CA* mutations have a frequency of 2% to 5% in NSCLC and are usually more prevalent in SCC (145). In adenocarcinoma, *PIK3CA* alterations may indicate a worse prognosis and have also been described as concurrent with other oncogenic drivers like *EGFR*, impacting on sensitivity to TKIs (146).

Conclusions

Precision medicine has greatly improved the management of patients with advanced lung adenocarcinomas (1). In this context, molecular characterization is crucial to assess the best therapeutic options both in first and further line settings (6). Several predictive biomarkers have been introduced in clinical practice, and there is still an increase in the number of variants that should be routinely characterized (16).

Many targetable alterations in oncogenes have well known clinical implications, but some aspects related to the molecular landscape of lung cancer need to be better clarified. It has been demonstrated that the single oncogenic driver paradigm is not completely appropriate within the context of lung cancer. Tumor heterogeneity and co-occurring alterations, especially in oncosuppressor genes like *TP53* or *STK11*, can define subgroups among lung cancers addicted to the same oncogene, with different responses to therapies

and tumor microenvironment (10). The identification of resistance mechanisms can be challenging, and the selection of patients eligible for immunotherapy is still a debated topic. In this regard, sex differences contribute to peculiar molecular characteristics. Molecular differences between sexes are mainly due to tobacco exposure and hormonal status and lead to different frequencies of driver alterations, response to therapy and prognosis (114,115). A higher incidence of targetable driver alterations has been reported in non-smoker women compared to men, and sex-specific alterations in genes involved in tobacco metabolism have been identified (114). Moreover, hormonal status influences cancer development and progression and can be associated with the activation of targetable pathways. Different responses to TKIs between men and women harboring the same driver alterations have been highlighted; susceptibility to immunotherapy is also different between sexes (11,17).

Although interesting data are already available, the basis of molecular differences between sexes is not fully understood and further studies are necessary to evaluate the predictive values of sex-related molecular features in clinical practice.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Editta Baldini and Franca Melfi) for the series "Lung Cancer In Women: From Epidemiology To Therapy" published in *Precision Cancer Medicine*. The article has undergone external peer review.

Reporting Checklist: The authors have completed the Narrative Review Reporting Checklist. Available at http://dx.doi.org/10.21037/pcm-20-72

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/pcm-20-72). The series "Lung Cancer In Women: From Epidemiology To Therapy" was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/pcm-20-72
Cite this article as: Bruno R, Alì G, Poma AM, Fontanini G. Non-small cell lung cancer molecular characterization of advanced disease with focus on sex differences: a narrative review. Precis Cancer Med 2021;4:14.