Heterogeneity in Lung Cancer

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Keywords

Lung cancer · Heterogeneity · Molecular pathology · Genetics

Abstract

Lung cancer diagnosis is a challenge since it is also one of the most frequently diagnosed cancers. Diagnostic challenges are deeply related to the development of personalized therapy and molecular and precise histological characterizations of lung cancer. When addressing these features, it is very important to acknowledge the issue of tumour heterogeneity, as it imposes several questions. First of all, lung cancer is a very heterogeneous disease, at a cellular and histological level. Cellular and histological heterogeneity are addressed with emphasis on the diagnosis, pre-neoplastic lesions, and cell origin, trying to contribute to a better knowledge of carcinogenesis. Molecular intra-tumour and inter-tumour heterogeneity are also addressed as temporal heterogeneity. Lung cancer heterogeneity has implications in pathogenesis understanding, diagnosis, selection of tissue for molecular diagnosis, as well as therapeutic decision. The understanding of tumour heterogeneity is crucial and we must be aware of the implications and future developments regarding this field.

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Introduction

Lung cancer is one of the most frequently diagnosed cancers, especially in developed countries. Despite recent developments in the diagnosis, classification, and therapy, the overall survival is still poor. Better understanding of the pathology of these tumours, especially molecular pathology, is necessary to accumulate knowledge in order to better address this issue, aiming at personalized therapy.

Tumour heterogeneity has been described in several tumours and it has been addressed in several ways: histological, cellular, and molecular/genetic. Lung cancer constitutes a group of heterogeneous tumours, with several differentiation types, recognized by the WHO classification of lung tumours. In this classification, the importance of molecular characterization of lung cancer is recognized.

Tumour heterogeneity has an important impact not only on tumour classification but also on defining prognosis and therapy decision. So, it is crucial to understand the implications of tumour heterogeneity in daily diagnosis routine.

The authors aim to address tumour heterogeneity in lung cancer, exploiting histological, cellular and molecular heterogeneity. They intend to review the heterogene-

ity issue, discussing and reflecting on lung heterogeneity and addressing the new WHO classification and several published knowledge.

Histological and Cellular Heterogeneity in Lung Cancer

Lung cancer is a heterogeneous group of cancers. Improvements in the histological classification are continuously being made. The new WHO classification of lung tumours has recently been published. This classification takes into account the great histological heterogeneity in lung cancers. It recognizes several types of lung cancers, such as epidermoid carcinomas, adenocarcinomas, small cell lung carcinomas, large cell carcinomas, large cell neuroendocrine carcinomas, adenosquamous carcinomas, sarcomatoid and pleomorphic carcinomas, and several other types. Non-small cell lung cancer reporting is not acceptable without an immunohistochemical profile to achieve differentiation as well as to identify genetic alterations and biomarkers, some with predictive value for therapy decision. Small biopsies are prone to identify CK7/TTF1, CK5.6/p40, and vim/neuroendocrine markers. Biopsies keep being the most representative tissue to classify and treat pulmonary carcinomas due to the high percentage – 70% – of tumours diagnosed in a non-surgical way.

This great variety of histopathologic diagnosis reflects tumour heterogeneity that could be explained by different cells of origin or differentiation pathways.

Even in carcinomas like squamous cell carcinomas or adenocarcinomas heterogeneity has been found, as histological subtypes have been defined. For instance, variants of squamous cell carcinoma are recognized. The same is true for adenocarcinomas. These histopathological subtypes have diagnostic, prognostic, therapeutic and demographic distinct features. So, although similar in differentiation, they represent different tumours, thus reinforcing that heterogeneity is a feature of these tumours.

Histological and cellular heterogeneity in lung cancer is also well proven when we look for tumours with more than one type of differentiation. Adenosquamous carcinomas are a good example. They clearly demonstrate heterogeneity at the cellular level as we can find cells with adenocarcinoma differentiation markers like CK7 and TTF1 as well as with squamous differentiation markers such as CK5/6 or other high-molecular-weight cytokeratins. This is also true for pleomorphic carcinomas where we can find areas with squamous or adenocarcinoma differentiation and giant and/or fusiform cells.

Cellular heterogeneity is also observed in combined tumours such as small cell lung carcinomas combined with other lung carcinomas such as adenocarcinoma or squamous cell carcinoma or large cell neuroendocrine carcinomas combined with other carcinomas.

This cellular heterogeneity could be, at least in part, explained by different cell origins. Pre-neoplastic lesions for some lung carcinomas are defined.

Two repair/carcinogenic pools of adult stem cells have been reported and are related to pulmonary carcinoma heterogeneity: the TRU – terminal respiratory unit; i.e., the respiratory bronchiole and adjacent alveolar duct/septae – and the adult central respiratory epithelium till the TRU. Embryonic development of human-induced pluripotent stem cells after induction of adult fibroblasts in mouse skin has demonstrated the whole embryonic potential informing about the repair/adaptation and the possibilities of the intermingling of meso/ecto/endoderm in one single cell. In this preliminary study, CK7, CK5.7, TTF1, VIM, CD56, and Ki-67 demonstrated to be enough to classify carcinomas according to cellular populations/heterogeneity. Similar results have been obtained with chromium treatment of both fibroblasts and normal bronchial cells in contact where epithelial-mesenchymal transition was revealed.

It is well known that squamous cell carcinomas have a basal cell origin (in the respiratory epithelia), demonstrated by our group and several other investigators. The sequence basal cell hyperplasia – squamous metaplasia – squamous dysplasia – squamous carcinoma is well defined. Our research group also demonstrated molecular changes in this spectrum of lesions, reflecting the severity and biological aggressiveness along the spectrum of lesions, using immunohistochemical markers such as Ki-67, p53, EGFR, and HER2 and also evaluating EGFR and HER2 gene copy number [1].

We have studied EGFR expression in these pre-neoplastic lesions, demonstrating its increasing expression in lesions in this spectrum; therefore, EGFR and its respective pathways may play a role in early steps of epidermoid carcinoma development, reflecting the importance of EGFR signalling transduction pathways in pre-neoplastic lesions [1]. Other studies also corroborate these results [2–9]. On the other hand, it seems that HER2 might not be involved in the first steps of epidermoid cancer development; other studies have found identical results [8, 10, 11].

EGFR, Ki-67, and p53 might play a role in the identification of epidermoid carcinoma pre-neoplastic lesions at higher risk of developing epidermoid carcinoma [1].

An increasing expression of these markers was observed [1]. The study reinforced the utility of Ki-67 as a biomarker for dysplasia, a group of pre-neoplastic lesions characterized by a higher proliferative index, also identified by other authors [1, 12–17].

Atypical adenomatous hyperplasia is considered as an adenocarcinoma pre-neoplastic lesion. Peripheral adenocarcinomas have their origin in epithelial cells of the TRU, like pneumocytes, with CK7 and TTF1 expression. Nevertheless, we must consider that more central adenocarcinomas have a different cell of origin as they develop upstream the TRU. So, it is hypothesized that the origin is in cylindrical cells of the respiratory tract. Also, adenocarcinomas can clearly have a lung differentiation demonstrated by CK7 and TTF1 positivity, but some lung adenocarcinomas express CK20 and are TTF1 and CK7 negative, revealing a colonic-like expression. Another perspective is to observe the way cells are organized. Several patterns of cellular organization can be observed. These different patterns may reflect not only cellular type/differentiation but molecular differences that could explain cellular organization and even biological behaviour, as well as prognostic and therapeutic differences in between patterns. Our research group has been addressing lung carcinomas considering histological patterns, especially in adenocarcinomas but also exploring adenosquamous and pleomorphic carcinomas [18]. For instance, we proved the relevance of pattern classification demonstrating that adenocarcinomas are molecularly different from normal adjacent tissue, and that acinar and BA/lepidic patterns are the most alike and papillary patterns the most different [18]. Cluster analysis revealed three clusters: papillary, solid, and a group composed of acinar, BA/lepidic and micropapillary patterns [18]. Papillary and solid patterns revealed lower TTF1 expression (identical to normal tissue), exhibiting a non-TRU/bronchial phenotype [18]. Acinar, BA/lepidic and micropapillary patterns showed higher TTF1 expression corresponding to TRU origin [18]. These patterns, especially lepidic and acinar, being TTF1-positive, are those where EGFR mutations are said to be more frequent [18]. TTF1 expression, identifying possible TRU origin, defines a subgroup of adenocarcinomas with molecular and biological particularities [18]. The solid pattern also revealed lower HER2 and higher EGFR and ERCC1 expression (compared to papillary) [18]. In solid patterns, EGFR pathway activation was related to EGFR overexpression [18]. We stated that adenocarcinomas with a solid pattern were less differentiated adenocarcinomas, with a worse prognosis [18]. Papillary patterns showed higher HER2 and lower

ERCC1 expressions. It seems that impaired DNA repair mechanisms are implicated in carcinogenesis when the papillary pattern is dominant [18]. Adenocarcinomas showed higher TTF1 expression in acinar, BA/lepidic and micropapillary patterns corresponding to TRU adenocarcinomas that express TTF1 [18, 19], probably related to a better prognosis when compared to non-TRU-type adenocarcinomas [20]. This is also highlighted by some authors that identified an inverse correlation between TTF1 and Ki-67, a marker of proliferation and biological aggressiveness [21]. We identified higher TTF1 expression in acinar and lepidic patterns, in TRU-type adenocarcinomas, corroborating the published results [18]. TTF1, expressed in TRU-type adenocarcinomas, has been associated with good prognosis [21–26].

However, Pelosi et al. [27] did not find a correlation between TTF1 expression and prognosis. Several studies identified a correlation between TTF1 expression, like TRU-type adenocarcinomas, and EGFR mutations [28, 29]. TRU-type adenocarcinomas have also been associated with EGFR mutations [28–32]. It is also known that EGFR mutations are more frequent in lepidic and acinar patterns. EGFR protein expression has been described as more frequent in TRU-type adenocarcinomas as well as in epidermoid lung carcinomas [33].

The micropapillary pattern had higher retinoblastoma protein (RB) expression, and the acinar pattern lower ERCC1 and higher EGFR expression when compared with normal tissue [18]. Cyclin D1 seemed to be relevant in acinar and BA/lepidic patterns and not related to the micropapillary pattern [18].

ERCC1 protein expression in micropapillary, solid and BA/lepidic patterns indicated DNA repair preservation, while in acinar and papillary patterns, there was lower expression [18]. Lung cancer with higher ERCC1 expression was associated with cisplatin-based chemotherapy resistance as ERCC1 acts by removing DNA adducts, which relates to poor prognosis [34–39].

These differences identified between the adenocarcinoma patterns represent a form of heterogeneity with implications in the diagnosis, pathogenic understanding, and therapeutic outcome.

We can also argue that carcinogens could have a role in determining heterogeneity as some lung carcinomas are more frequently diagnosed in smokers or ex-smokers, such as small cell lung carcinoma and squamous cell carcinoma, and some other carcinomas, like adenocarcinoma, are being diagnosed in never-smokers. However, there are adenocarcinomas related to smoking and others not related to smoking. This fact indicates that the envi-

ronment is also a determinant of tumour heterogeneity, as different exposure histories are related to different genetic and molecular changes, explaining the histological heterogeneity. Also, we know that several lung pathologies/conditions are associated with an increased risk of lung carcinoma, such as lung fibrous/scar areas and lung interstitial diseases. These diseases could constitute a field of cancerization, and some may share molecular pathways with the lung cancers, such as pathways implicated in epithelial mesenchymal transition (EMT).

Tobacco and professional or other exposure inhaling demand cellular remodelling and adaptation of the basal cells in either TRU or respiratory epithelium, with consequent hyperplasia of vimentin-positive cells and TTF1 bronchial-positive cells, demonstrated in carcinomas arising after molecular transformation in less matured cells, and consequently, in multi-patterned carcinomas and in pleomorphic carcinomas. This approach facilitated pulmonary carcinoma classification in biopsies but does not correlate directly with metastatic potentiality.

Histological heterogeneity is also observed in the differentiation grade. For instance squamous cell lung carcinoma could be classified as well or poorly differentiated. The degree of differentiation is associated with a greater biological aggressiveness and poor outcome.

So, only by looking for cellular and histological heterogeneity can we say that lung carcinomas are a good model for cancer heterogeneity study. Cancer heterogeneity is a constant and identified in other organs and system of organs.

Several authors recognize morphologic heterogeneity in lung adenocarcinomas and the WHO recommends reporting the patterns present as well as the most prevalent pattern. These patterns have prognostic value, with the lepidic pattern having better prognosis and the micropapillary and solid pattern having worse prognosis. This morphologic heterogeneity also has therapeutic implications as patients with solid and micropapillary patterns benefit more from adjuvant chemotherapy [40–42].

We also know that different molecular expression patterns are associated with heterogeneity as well as with prognosis. The differential expression of several molecular markers, such as c-erb-2, bcl-2, p53, p63, rb, egfr, and neuroendocrine markers, reinforces the utility of pattern-based classification [40, 41].

Morphologic heterogeneity has clear prognostic implications. We clearly know that the histological type is associated with prognosis (for instance small cell carcinoma has worse prognosis and in situ adenocarcinoma and typical carcinoid have better prognosis). Also, the

histological subtype is associated with prognosis (for instance, well-differentiated squamous cell carcinoma compared to basaloid squamous cell carcinoma and typical carcinoid compared to atypical carcinoid) [42–45].

We all also recognize that morphologic or histologic heterogeneity has therapeutic implications especially as the prevalence of molecular targetable changes differs in between histologic subtypes [42, 45, 46]. For instance, *EGFR* mutations are more frequently identified in lung adenocarcinoma, especially arising from the TRU, and also more frequently identified in some patterns like lepidic, papillary and acinar patterns [45, 47]. *ALK* translocations are more prone to be identified in lung adenocarcinomas and in acinar, solid and signet cell morphology [45, 48–50]. However, there are some conflicting results as some authors identify *EGFR* mutations in all the patterns of lung adenocarcinomas; these authors identified an association of *KRAS* and *BRAF* mutations and high nuclear grade [51].

Molecular heterogeneity has been identified in morphologic heterogeneous carcinomas such as in adenocarcinomas, for *KRAS* mutations and *EGFR* mutations [52]. Molecular heterogeneity related to *KRAS* mutations status has also been identified in pleomorphic carcinomas [53]. By whole-genome sequencing, different rates of gene mutations, gene copy number alterations, and different protein expression levels and protein phosphorylation levels were demonstrated, confirming the association between morphologic and molecular heterogeneity [54]. Fang et al. [54] reported that p53 is the most frequently mutated gene; *KRAS*, *EGFR*, *MLL3*, and *STK11* the most frequently mutated genes in adenocarcinomas; *PI3KCA*, *SOX2*, *CDK2*, *P63*, and *FGFR1* the most frequently mutated genes in squamous cell carcinomas; and *RB1*, *MLL2*, *SMO*, and *PI3KCA* the most frequently mutated genes in small cell lung cancer.

Molecular and Genetic Heterogeneity

Tumour molecular and genetic heterogeneity has been identified not only in lung tumours but also in other organs. Molecular/genetic heterogeneity has been identified in breast, gastric, bladder, prostate, pancreatic, as well as in lung cancer [55–57].

Only 1/3 of somatic mutations are present in all the regions of the same tumour [57]. Intra-tumour heterogeneity (ITH) was first described by Fidler [58].

Genetic heterogeneity has been described in lung cancer [59–63]. Tumour heterogeneity has been explained by

genetic heterogeneity (intra- and inter-tumour heterogeneity) and also by non-genetic heterogeneity driven by external and internal pressures allowing outgrowth of cell subpopulations, some cases depending on selective pressure related to microenvironment and interactions with immune and stromal cells or with matrix components [61, 62, 64, 65].

Molecular heterogeneity could be explained by several mechanisms such as by stem cell theories, by genomic or chromosomal instability, epigenetics modifications, and by adaptation mechanisms in response to microenvironment stimulus [66]. This molecular/genetic heterogeneity is associated with resistance to therapy like EGFR-targeted therapy [66].

ITH is also explained by clonal (monoclonal or polyclonal) evolution resulting in genetic heterogeneity, by selective pressure induced by the microenvironment or by chemotherapy favouring one or more than one clone of cells, by the EMT theories and by inter-clonal cooperation mechanisms [61, 67–69]. Inter-clonal cooperation is also important for metastases, as clones favour metastases by sequential or simultaneous cooperation in motility, matrix degradation, vascular invasion or distant colonization [67, 70–74].

Other authors associate tobacco with ITH, as they identified *EGFR* mutation heterogeneity according to adenocarcinoma pattern (more frequent in lepidic pattern) and to tobacco habits [75]. Multiple carcinogens present in tobacco are related to the development of a field of cancerization, where cells tend to accumulate several molecular changes leading to genetic instability and therefore favouring heterogeneity. Other authors identified associations between gene copy numbers (for *EGFR*) and intra-tumour mutation heterogeneity [76]. These authors propose that non-small cell lung cancer could be stratified into four groups according to ITH: pure mutated; pure wild-type, mutated heterogeneous; and wild-type heterogeneous. Those tumours with higher or pure mutational rates mutated with better response to EGFR-targeted therapy [76].

DNA repair has important implications in cancer. Deficient DNA repair function allows genomic instability [77, 78]. Genomic instability allows the accumulation of mutations thus promoting heterogeneity. DNA repair impairments could also explain the increased sensitivity of tumour cells to radiation and chemotherapy and thus clonal selection pressure [77, 78]. DNA damage could be related to spontaneous hydrolysis, cytosine deamination, mismatched bases, and secondary to reactive oxygen species (ROS) [77, 78]. Also, anticancer agents, such as alkyl-

ating agents and bleomycin, are responsible for DNA breaks [77, 78]. Six different mechanisms are involved: mismatch repair, homologous recombination and non-homologous end joining, translesion DNA synthesis, base excision repair, and nucleotide excision repair [77, 78].

Several authors and studies revealed molecular/genetic heterogeneity concerning *EGFR* and *KRAS* mutations; however, conflicting results have been published [79]. Bai et al. [76] identified an ITH rate of 28.2% (24/85%); Mansuet-Lupo et al. [80] identified an ITH rate of 5% (2/40); Kim et al. [81] an ITH rate of 2.9% (1/34); Tomonaga et al. [75] an ITH rate of 23.7% (9/38); Taniguchi et al. [82] an ITH rate of 28.6% (6/21); Zhang et al. [83] an ITH rate of 100% (7/7); Zhong et al. [79] found an ITH rate of 15.4% (10/65), mainly in adenocarcinomas and in some studies in squamous cell carcinomas and adenosquamous carcinomas. On the other end, other studies and authors did not find ITH [29, 84–86]. Some of these works had small samples and studied less foci of the same tumour compared to the studies where ITH was identified. Other authors showed that *EGFR* mutation heterogeneity is rare in primary tumours and metastasis, being higher in multiple lung nodules, with ITH rates of 9.1%, discordance rate between primary and lymph node metastasis of 10.2%, discordance rate of 14.3% between primary and distant metastasis and discordance between multiple lung nodules of 24.4% [87].

Zito Marino et al. [88] identified ITH for *ALK* translocation but no ITH for *EGFR* in lung adenocarcinomas.

Several authors (Badalian et al. [89], Cortot et al. [90], Kalikaki et al. [91], Schmid et al. [92], and Sun et al. [93]) identified ITH for *KRAS* mutation status in adenocarcinomas, ranging from 69 to 86%. However, Alsdorf et al. [94] reported that intra-tumour *KRAS* heterogeneity is a rare event, without discordance between primary tumour and metastasis.

For *MET* high intra-tumour spatial heterogeneity rate was identified in non-squamous lung carcinomas, associated with worse prognosis [95].

Taking into account that most of the authors report intra-tumour genetic/molecular heterogeneity, we could ask if the small biopsy is representative of the whole genetic panorama of the neoplasia. As the concordance rate varies between 68 and 97.1% for *EGFR* mutation status, we could argue that according to our group reports for most cases biopsy tissue will be representative, but not in some other cases [75, 76, 79–83, 96]. On the other end, Zhong et al. [79] and Yatabe et al. [84] stated that *EGFR*, *KRAS*, and *ALK* ITH is rare, defending the representativeness of the tissue obtained by biopsy. Mansuet-Lupo et al.

[80] also showed that *EGFR* mutations are independent of the primary tumour localization, of the type of sample, and consistent between primary and metastasis, validating the use of biopsies. Intra-tumour genetic heterogeneity for *EGFR* or *ALK* could also explain different TKI response rates between patients. Clonal selection and acquisition of new mutations could also be responsible for resistance. Re-biopsy or metastasis biopsy is important to evaluate therapeutic resistance. As some patients do not have conditions to re-biopsy or have inaccessible tumours, liquid biopsy is gaining importance and actually is a valid tool for diagnosis, evaluation of response, and recurrence as well as to address spatial and temporal heterogeneity.

Temporal genetic/molecular heterogeneity is also being studied in lung cancer. This could be related with temporal heterogeneity in the primary tumour or between primary tumour and metastasis over time. This temporal heterogeneity is of great importance in the understanding of recurrence and therapeutic resistance. Kim et al. [97], by next-generation sequencing, identified infrequent genetic heterogeneity of 16 genes between primary tumour and metastasis. Sherwood et al. [98], in a review of 26 articles, demonstrated that there are variable discordance rates between primary tumour and their metastases. However, as there is a substantial concordance, the molecular diagnosis could be made in the primary tumour or in the metastasis, recommending, however, the use of sensible methods [98]. The concordance or discordance could be explained by sample issues as the percentage of tumour cells, by the methodologies applied on diagnosis, by ITH, temporal heterogeneity (mutational status evolution), and preservation methods and even by the local of metastasis [98]. *EGFR* concordance rate between primary tumour and metastasis ranged from 100 to 72% [98]. Kalikaki et al. [91] identified concordance rates of 72% (18/25 cases), Schmid et al. [92] 94% (90/96), Mansuet-Lupo et al. [80] 90% (9/10), Sun et al. [93] 91% (73/80), Wei et al. [105] 94% (47/50), Yatabe et al. [84] 100% (77/77), Shimizu et al. [104] 86% (60/70), Park et al. [103] 88% (89/101), Matsumoto et al. [102] 100% (8/8), Luo et al. [101] 93% (14/15), Gow et al. [99] 73% (49/67), and Han et al. [100] identified a concordance rate of 81% (30/37). *KRAS* concordance rate between primary tumour and metastasis ranged from 100 to 64% [98]. *KRAS* concordance rates identified by different authors were: 76% (19/25) [91]; 74% (71/96) [92]; 93% (74/80) [93]; 100% (9/9) [94]; 100% (15/15) [106]; 64% (7/11) [89]; 71% (15/21) [90]; 81% (17/21) [90] (using ARMS); and 97% (36/37) [100].

Lung cancer heterogeneity raises prognostic questions. Recurrence after therapeutic resistance is one of

the most important causes of cancer-related death. Resistance mechanisms are related to signal transduction-redundant activation, new mutations, synergic interactions with the target gene, *EGFR* inhibition bypass, EMT phenotype acquisition, DNA hypermethylation, and also related to the emergence of new tumour cell subclones with secondary mutations resistant to previous therapy, this event is related to selective pressure and heterogeneity (cellular and molecular) [66, 107, 108]. Therapeutic selective pressure is associated with *EGFR* TKI resistance [66, 107, 108]. Chemotherapy and targeted therapies are associated with the reduction of the number of sensible cell clones and to a higher proportion of resistant clones that persists after treatment. After chemotherapy, the response rate to *EGFR* TKIs is lower and some authors report a decrease in *EGFR* mutation rate [76, 109, 110]. Chen et al. [87] also demonstrated higher intra-tumour and inter-tumour heterogeneity in tumours of patients submitted to chemotherapy. Resistance mutations and amplification rates are higher before *EGFR* TKIs [66]. *EGFR* TKIs resistance takes place about 10–13 months after *EGFR* TKIs, related frequently to *EGFR* T790M mutation, *MET* amplification, *HER2* mutation, and *KRAS* mutation. Anti-*ALK* therapy resistance is often related to *ALK* mutations and amplifications, *KIT* amplification and *EGFR* activation. Bai et al. [76, 109] showed that patients with tumours showing higher *EGFR* gene copy number had lower ITH and those patients with higher heterogeneity had a worse prognosis and survival rate. Also they stated that tumours with higher *EGFR* mutation rates showed better *EGFR* TKI response [76, 109]. Taniguchi et al. [82] also demonstrated that time to progression and overall survival after *EGFR* TKIs is significantly lower in patients with tumours with high ITH.

As expected intra- and inter-tumour genetic and molecular heterogeneity have important therapeutic implications. We must also take into account that molecular heterogeneity is also reflected when we consider that some genes could have different mutations or genetic events, some conferring different therapeutic sensibility, as described for *EGFR* mutations where we can also find resistance conferring mutations, like T790M *EGFR* mutation [111]. Other *EGFR* mutations are associated with less sensibility to TKIs (exon 18 G719 and exon 21 L861Q for instance), and exon 20 in frame insertion are associated with no affinity for TKIs [111].

ITH has also been identified at a metabolic level, some with prognostic significance and implications in disease progression after chemotherapy [112–115].

Another important issue is related to molecular heterogeneity when dealing with primary and metastatic disease. Several reports address this problem [58, 93, 98, 104, 116–118]. Temporal heterogeneity is also an issue to consider, with clinical implications in the clinical follow-up, determining the risk of recurrence and metastases, related to acquiring resistance to chemotherapy or targeted therapy [108, 109, 119–122]. Several authors have been addressing these issues.

To address the questions imposed by spatial and temporal heterogeneity including heterogeneity between primary tumour and metastasis and by the selective therapeutic pressure, several authors are recommending the sequential sampling of tumour cells or genetic material. This could be achieved by liquid biopsy either addressing circulating tumour cells or cell-free DNA like ctDNA. These approaches are useful in the selection of the treatment, monitoring and evaluation of early recurrence and minimal residual disease, and resistance acquisition [123–125]. This methodology is also relevant when patients show no tolerance to a new biopsy, if there are several metastases in different localizations, when the tissue is insufficient or has artefacts related for instance to decalcification, when there are problems related to heterogeneity or when biopsy imposes risks [124]. ctDNA can be used to identify resistance to TKIs [126–135]. Weber et al. [136] demonstrated a concordance rate of 90% (179/199) for *EGFR* mutations between biopsy tissue and ctDNA (plasma) before *EGFR* TKIs. These authors identified *EGFR* mutations in the plasma not identified in the biopsy, probably related to sampling issues or heterogeneity [136]. Douillard et al. [137] demonstrates a concordance rate of 94.3% between tumour samples and plasma ctDNA. Mok et al. [138] achieved a concordance rate of 88%. Kim et al. [139] identified a concordance rate of 87.7% and 5 cases (8.7%) with *EGFR* or *KRAS* mutations only in plasma. However, some studies showed cases where the mutational status evaluated in the plasma did not totally represent the mutational status in the tumour [108]. Tissue is still the choice when it possible to biopsy. In negative cases, it is necessary to recur to tissue and to apply more sensitive methods. Tissue biopsy is still the gold standard, but liquid biopsy could be adequate specially to overcome the problems related to biopsies.

Genetic/molecular heterogeneity is recognized when we consider the most frequent mutations according to the histologic type. *EGFR*, *KRAS*, *P53* mutations and *ALK*, *RET*, *ROS1* rearrangements and *EGFR* and *MET* amplifications are more frequently identified in lung adenocar-

cinomas [77, 140–146]. *P53*, *PI3KCA* mutations and *FGFR1* amplification are more frequently identified in lung squamous cell carcinomas [77, 145, 147–150]. *RB* and *P53* mutations and *MYC* amplification are more frequently identified in small cell lung cancer [42, 77, 141, 151].

Our studies in bronchial-pulmonary carcinomas, combining immunohistochemical and molecular pathology testing, have demonstrated the following cascades in pulmonary carcinomas: epidermoid carcinoma – *EGFR* and *HER2* polysomy and *CK7/Vimentin* for EMT non-pure epidermoid carcinomas; bronchial-pulmonary adenocarcinomas – non-smoking females – mutated *EGFR* and *ERCC1* expression; micropapillary pattern with *VIM/RB/ERCC1* expression; acinar/BA-lepidic/micropapillary patterns express *TTF1* and mutated *EGFR* [18, 47, 152].

We verified that generally *EGFR* mutations were present in all the patterns of the same adenocarcinoma, reinforcing the possibility of mutational status determination in biopsies [47]. Some other authors also identified that the identification of *EGFR* mutations was independent of the localizations in the primary tumour and concordant with metastasis [80]. However, we did find some cases where the mutations were not present in all the patterns [47]. We also found cases with harbouring different *EGFR* mutations in different patterns, nevertheless all mutations were activating mutations [47]. We have also identified cases with more than one type of *EGFR* mutation in the same patterns/cells of the same pattern and even have published cases with *EGFR* and *KRAS* mutation coexistence [47]. These facts are in favour of the tumour heterogeneity hypothesis as these complex *EGFR* mutations (coexistence of more than one type) were detected in adenocarcinomas, clearly demonstrating a molecular complexity that might be related to different cell clones or genomic instability responsible for the accumulation of multiple molecular events in the *EGFR* gene.

Our group has also investigated DNA promoter hypermethylation according to histologic type. We also found heterogeneity when studying *MHL1* and *MSH2* gene methylation [153]. A higher prevalence of the *MLH1* gene was identified mainly in squamous cell carcinoma (72%) [153]. However, no obvious differences were found for *MSH2* promoter hypermethylation [153].

FGFR1 was recently considered as a driver oncogene, especially for squamous cell carcinoma [154–156]. Some authors have identified overexpression and increased gene copy number especially in squamous cell carcinomas, an example of inter-tumour heterogeneity.

In our research, we have evaluated the FGFR1 gene, not only in squamous cell carcinoma but also in other histological types. We found FGFR1 protein expression in all subtypes of lung bronchial-pulmonary carcinomas, especially in pleomorphic carcinomas [157]. FGFR1 amplification, although more frequent in squamous cell carcinoma, was also identified in adenocarcinomas, adenosquamous and pleomorphic carcinomas [157]. Higher expression in pleomorphic carcinomas suggests that overexpression may also be implicated in the activation of the EMT pathway [157]. Overexpression could also be responsible for tumour growth and proliferation and invasiveness, related with a more aggressive behaviour [157].

Inter-tumour heterogeneity can also be identified at a metabolic level as some works of our groups of research demonstrate [158–162]. Distinct metabolic signatures have been found between lung adenocarcinomas between adenocarcinomas and squamous cell carcinomas. These findings could be identified in several biological specimens as neoplastic tissue, plasma, and urine [158–162]. The authors argue that RMN-based technologies could be used for diagnostic purposes after validation [158–162].

The development of next-generation sequencing methods allowed to easily demonstrate intra-tumour and inter-tumour genetic heterogeneity.

Thus, it is very important to know that lung cancer heterogeneity is a fact, with implications in pathogenesis understanding, carcinogenesis, pathological diagnosis, selection of tissue for molecular diagnosis, and in therapeutic decision. The understanding of tumour heterogeneity is crucial and we must be aware of the implications and future developments regarding this field.

Tumour heterogeneity could be addressed by different perspectives; for the pathologist as histological or pattern differences in the tumour, for the molecular pathologist as genetic/molecular and epigenetic variations in the tumour (spatial and temporal), and for the oncologist as heterogeneity related to therapeutic response and resistance.

Tumour heterogeneity is changing the paradigm: initially one treatment was fitted to all patients, then, with the emergence of targeted therapies, one patient/one treatment was applied, and now we are heading towards precision medicine, i.e., one patient/one moment in the disease evolution/one treatment.

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