Clonality of multifocal nonsmall cell lung cancer: implications for staging and therapy

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ABSTRACT: Nonsmall cell lung cancers (NSCLCs) display a variety of morphological and molecular features. Accurate subtyping of NSCLC has been shown to predict patient survival as well as response rates and toxicities of specific drugs. Assessment of multifocal lung tumours and the distinction of synchronous primary tumours from intrapulmonary metastases represent an important problem as this decision significantly influences tumour staging and subsequent treatment strategies.

In order to provide a basis for evidence-based treatment decisions in these patients, we analysed the clonal relationship of multifocal NSCLC with indistinguishable histomorphology in a series of 78 patients by allelotyping (using polymorphic short tandem repeat markers) as well as *KRAS* and epidermal growth factor receptor (*EGFR*) mutation testing.

Our data demonstrate a common clonal origin indicative of intrapulmonary metastases in almost two-thirds (~62%) of the cases, while ~36% of multifocal NSCLC displayed unique molecular profiles suggesting separate primary tumours. Divergent *KRAS* and/or *EGFR* mutations were observed in ~8% of all cases.

With the increased availability of *EGFR*-targeted therapy options, nonresectable, multifocal NSCLC with diverging *KRAS* and/or *EGFR* mutations are likely to show different treatment responses, underlining the need to separately analyse multifocal tumours. Obviously, this also holds true for further, novel molecular predictors of targeted therapies.

KEYWORDS: Adenocarcinoma, clinical lung cancer, loss of heterozygosity analysis, lung cancer chemotherapy, lung cancer diagnosis, thoracic oncology

Lung cancer is the leading cause of cancer-related mortality in developed countries [1]. Approximately 80% of these tumours are nonsmall cell lung cancers (NSCLCs). Recent translational research in large multicentre trials demonstrated that histological and molecular subtyping of NSCLC is of clinical relevance, not only regarding patient survival but in predicting drug response and toxicity [2]. While adenocarcinomas (ADCs) harbouring activating epidermal growth factor receptor (*EGFR*) mutations are associated with responsiveness to tyrosine kinase inhibitors (TKIs) and show a significantly better progression-free survival, patients without *EGFR* mutations receiving TKI treatment have a substantially poorer outcome compared with conventional cytotoxic chemotherapy [3]. Thus,

careful histopathological evaluation combined with predictive molecular analysis is of paramount importance to optimise therapeutic strategies and treatment in NSCLC patients.

The frequency of multiple anatomically separate lung tumours of indistinguishable histology in NSCLC patients has been reported to range from 0.2% to 8% (3.5% to 14% in autopsy studies) [4–7]. The majority of those cases seem to be clonally related and surgical resection is considered in selected patients [8]. However, pathologists should attempt to distinguish multiple primary tumours from intrapulmonary metastases [7, 9]. This distinction not only influences tumour staging (according to the TNM (tumour, node, metastasis) system), but also subsequent

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predictive analyses such as *EGFR* mutation testing. In order to provide a basis for evidence-based treatment decisions in these patients, we analysed the clonal relationship of 78 patients with multifocal NSCLC lesions with indistinguishable morphology by allelotyping (using polymorphic short tandem repeat markers), as well as the mutational status of *KRAS* and *EGFR* by Sanger sequencing. We demonstrated that a common clonal origin of multifocal NSCLC is evident in the majority of cases. However, approximately one-third of multifocal lung tumours display clonally distinct molecular profiles, which may significantly influence response rates to tailored therapies.

PATIENTS AND METHODS

Tumour samples

We screened our archives for cases of synchronous, multifocal NSCLC either in the same lobe (pT3), in different lobes of the same side (pT4) or in different lobes of both sides (pM1a). Cases were thoroughly screened for histomorphological criteria of the nodules, which may help to distinguish between metastases and multiple primaries and were only included when the morphology was indistinguishable. Clinicopathological criteria for this were initially developed by MARTINI and MELAMED [10] and were further extended by other authors [11–13]. The most comprehensive approach using histomorphological criteria, which was also applied for this study, was developed by GIRARD *et al.* [14]. These criteria include grade, cytological features, stromal characteristics and relation to foci of carcinoma *in situ* as well as mucinous, fetal, colloid, signet-ring, clear-cell, lepidic, acinar, papillary, micropapillary and solid differentiation for ADC, and papillary, clear-cell, basaloid and sarcomatoid differentiation for squamous cell carcinoma (SQCC). In general, multifocal tumours are potentially considered as clonally related when the criteria are similar in the respective tumour nodules. All tumours were resected at the Thoraxklinik Heidelberg (Heidelberg, Germany) and diagnosed at the Institute of Pathology (University Hospital Heidelberg, Heidelberg), according to the current World Health Organization classification for lung tumours [15]. Usage of the tissue was approved by the local ethics committee (University Hospital Heidelberg, approval number 206/2005).

DNA isolation and mutation analyses

Haematoxylin- and eosin-stained slides from all NSCLC specimens were reviewed for appropriate regions with tumour cell concentrations >50%. Deparaffinised, 10-µm sections were used for manual microdissection with the help of a glass needle, followed by digestion using proteinase K (0.5 mg·mL⁻¹; Fermentas, St Leon-Rot, Germany) in 20 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl₂ and 0.1% sodium dodecylsulfate overnight. The enzyme was then heat inactivation at 95°C for 5 min.

For PCR amplification of *EGFR*, the following primers were used: 5'-GCTGAGGTGACCCTTGCTCTC-3' (exon 18 forward) and 5'-ACAGCTTGCAAGGACTCTGG-3' (exon 18 reverse); 5'-GCTGGTAACATCCACCCAGA-3' (exon 19 forward) and 5'-GAGAAAAGGTGGGCCTGAG-3' (exon 19 reverse); 5'-CATGTGCCCCCTCTTCTG-3' (exon 20 forward) and 5'-GATCCTGGCTCCTTATCTCC-3' (exon 20 reverse); and 5'-CCTCACAGCAGGGTCTTCTC-3' (exon 21 forward) and 5'-CCTGGTGTGAGGAAAATGCT-3' (exon 21 reverse). Exon

1 of *KRAS* was amplified with the primers 5'-GTGTGACATGTTCTAATATAGTCA-3' (exon 1 forward) and 5'-GAATGGTCCTGCACCAGTAA-3' (exon 1 reverse).

PCR fragments were controlled by agarose gel electrophoresis and purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). *EGFR* and *KRAS* mutation screening was performed using single-strand conformation polymorphism (SSCP) sensitive gel electrophoresis as described previously [16]. Samples with aberrantly moving bands were subjected to direct DNA sequencing with an ABIPrism 377 Sequencer (Applied Biosystems, Darmstadt, Germany) using the DYEnamic ET Terminator kit (GE Healthcare, Freiburg, Germany). The use of SSCP screening for *EGFR* mutation testing has previously been validated [17].

Loss of heterozygosity analyses

For loss of heterozygosity (LOH) analyses, fluorochrome-conjugated primers (Biomers, Ulm, Germany) were combined into five primer pools based on their optimal annealing temperatures. Pool A (D22S444-FAM, D16S402-TET and D11S1311-HEX) and pool B (D17S1818-FAM, D8S264-TET and D17S785-HEX) were annealed at 50°C, whereas pool C (D1S507-FAM and D16S518-TET), pool D (D9S1812-FAM, D17S1852-TET and TPO-HEX) and pool E (D16S539-FAM, D9S925-TET and D16S2624-HEX) were annealed at 55°C. After 30 PCR cycles, the products of pools A and C and pools B and D were combined, heat-denatured and analysed on a MegaBace 1000 DNA sequencer (GE Healthcare). PCR products of pool E were sequenced separately after identical pre-treatment.

Statistics

Overall survival was estimated using the Kaplan–Meier method, with a log-rank test to probe for significance. Statistical analyses were performed using the R software (University of Vienna, Vienna, Austria). *p*-values <0.05 were considered significant. Univariate survival data were tested for significance using the Mantel–Haenszel log-rank test and are presented as Kaplan–Meier plots. Hazard ratios and 95% confidence intervals were calculated using univariate Cox proportional hazard regression.

Based on the observed distribution and frequencies of each analysed variable in the whole series, individual clonality scores were calculated for each tumour pair by multiplying the likelihood of the observed findings for each informative variable. Thus, this clonality score defines the statistical error probability of a clonal or a nonclonal relationship. Cases in which divergent mutations or a LOH pattern were observed were considered nonclonal. For the rest of the cases, tumour pairs with clonality scores of <0.05 were considered clonal while in cases with scores between 0.5 and 0.05, clonality was assumed to be likely. Tumours with clonality scores of >0.5 were considered non-informative and excluded from further analyses.

RESULTS

Clinicopathological characteristics

We finally analysed synchronous, multifocal tumour nodules of 58 ADC and 20 SQCC patients with indistinguishable histology for LOH and the mutational status of *EGFR* and *KRAS*. All clinicopathological data are summarised in table S1.

58 patients were male and 20 patients were female. Mean age at resection was 64 yrs (range 41–84 yrs). The cohort included 59 patients with multifocal tumours in one lobe (pT3), 18 patients with multifocal tumours in different lobes of the same side (pT4) and one patient with two synchronous tumours in different lobes of both sides (pM1a). Three ADC patients developed clinically evident distant metastases (two brain metastases and one metastasis to the adrenal gland), which were not subjected to surgery. One patient had received neoadjuvant chemotherapy. The smoking status could retrospectively be retrieved for 72 (92.3%) patients (table S1). All SQCC patients were active or former smokers. Among the ADC patients, there were eight never-smokers and six patients with no information concerning smoking status. Mean overall survival for patients who were alive (n=44) at the point of this study was 29.8 months. Mean follow-up for all patients was 25.6 months (range 0.3–87.4 months).

LOH and mutational analyses are suitable to assess clonal relationship of synchronous NSCLC

An overview of all LOH and Sanger sequencing-based mutational data is given in figure 1. We analysed 14 polymorphic

microsatellite markers and considered the loss of one allele as a minimal criterion. Tumour nodules of 22 (28.2%) patients displayed a divergent LOH status, indicating a dichotomous (nonclonal) origin (14 (24.1%) out of 58 ADCs and eight (40%) out of 20 SQCCs). The findings are underlined by the clonality score, which takes into account the frequency of the characteristic value of the variables and is a direct measure (statistical probability) of the overall evidence for clonality. Overall, 97.4% of the specimens could be classified as clonal, likely clonal or nonclonal. Two ADCs (2.6%; cases 38 and 39), with clonality scores of >0.5, were considered noninformative.

Activating *EGFR* mutations were detected in both synchronous tumours of four patients; *SQCCs* did not harbour *EGFR* mutations. *KRAS* mutations occurred in 20 (25.6%) patients (17 ADCs and three *SQCCs*) but strikingly, in six patients, mutations of *KRAS* were restricted to only one tumour nodule. Only one patient showed *KRAS* mutations in both tumour nodules. A divergent mutational status of *EGFR* and *KRAS* was detected in one patient, resulting in one tumour nodule with a *KRAS* and one tumour nodule with an *EGFR* mutation (case 12; table S1 and fig. 1). Overall, combined allelotyping by LOH and analyses of the mutational status of *KRAS* and *EGFR*

FIGURE 1. Loss of heterozygosity data and the mutational status of *KRAS* and epidermal growth factor receptor (*EGFR*) in 78 cases of synchronous nonsmall cell lung cancer (green: no molecular alteration detectable; red: allelic loss or mutation; white: noninformative). Clonal and nonclonal relationships of the tumour nodules are indicated in green and red in the top row, respectively. Dark green indicates cases with the highest evidence for a clonal (c) relationship (clonality scores <0.05) and light green represents tumours with scores between 0.5 and 0.05 (likely clonal (lc)). Two noninformative (ni) cases are indicated in grey (cases 38 and 39). ADC: adenocarcinoma; SQCC: squamous cell carcinoma; T1: tumour nodule 1; T2: tumour nodule 2; nc: nonclonal.

FIGURE 2. Impact of clonality of multifocal lung tumour nodules on the overall survival (OS) of a) adenocarcinoma (n=56; p=0.159) and b) squamous cell carcinoma patients (n=20; p=0.47). The two noninformative cases (cases 38 and 39) were excluded from the Kaplan–Meier analyses.

provided evidence that in 28 (35.9%) out of the 78 cases (18 (31.0%) ADCs and 10 (50%) SQCCs), the two analysed tumours were not clonally related. These findings not only significantly influence tumour classifications (table S1) but also staging and, thereby, personalised chemotherapeutic strategies. Notably, 14 (77.8%) out of the 18 synchronous ADCs with evidence for a nonclonal origin were localised in the same lobe and in 11 (61%) of the cases, lymph node metastases were evident (table S1 and fig. 1). Among the 38 ADCs with evidence for a clonal origin (table S1 and fig. 1), 31 (81.5%) of the synchronous tumours were localised in the same lobe and in 25 (65.7%) out of 38 cases, lymph node metastases were evident. The 10 synchronous SQCC cases with evidence for a nonclonal origin (table S1 and fig. 1) were mainly located in the same lobe (60%) and in only two cases were lymph node metastases evident. Among the 10 SQCC cases with evidence for a clonal relationship (table S1 and fig. 1), seven (70%) out of 10 synchronous tumours were located in the same lobe and six (60%) out of 10 cases displayed lymph node metastases.

Since field cancerisation is associated with smoking habits, we specifically analysed mean number of pack-years in relation to the clonality status (fig. 1). Only patients with known smoking and pack-year status were considered. Patients with SQCC had significantly more pack-years than ADC patients (57.1 and 32.4 pack-yr on average, respectively; $p=0.0065$, Wilcoxon rank test). ADC patients with clonally related and clonally unrelated tumours had means of 31.7 and 33.9 pack-yr, respectively. SQCC patients with clonally related and clonally unrelated tumours had means of 54.4 and 60.8 pack-yr (table S1). This demonstrates that smoking is associated with the development of lung cancer and that more intense smoking may increase the risk of developing multifocal, clonally unrelated tumours. However, the higher number of pack-years in the clonally unrelated tumours was statistically nonsignificant (ADC, $p=0.673$; SQCC, $p=0.240$; Wilcoxon rank test).

Impact of clonality on patient survival

To assess the prognostic impact of clonality, patient survival was analysed separately for individuals with clonally related

and unrelated tumours. Cases 38 and 39 were excluded from survival analyses since their clonality statuses could not clearly be determined based on the molecular analyses. Among the clonally unrelated and clonally related ADC cases, 33.3% and 50% of the patients died, respectively. In SQCC, tumour-specific death was observed in 30% of the patients with nonclonal tumour nodules and in 60% of the patient with clonally related SQCC lesions. Although there was an obvious trend that patients with two clonally unrelated tumours have a better outcome, univariate survival analyses failed to reach statistical significance (fig. 2). Hazard ratios for poorer survival in clonally related lung tumours were 1.93 (95% CI 0.76–4.86; $p=0.159$) in ADC and 1.66 (95% CI 0.41–6.65; $p=0.47$) in SQCC.

DISCUSSION

Up to 8% of lung cancer patients present with two or more anatomically separate tumour nodules [4]. To ensure adequate treatment in nonresectable constellations, it is crucial to distinguish clonally related tumours, representing intrapulmonary tumour spread, from separate primary tumours. In the present study, we have examined clonality in the largest series of synchronous, histomorphologically indistinguishable NSCLCs to date by means of polymorphic satellite markers and the mutational status of *KRAS* and *EGFR*. We provide evidence that while a common clonal origin of synchronous NSCLC is evident in the majority of cases, approximately one-third of multifocal lung tumours display clonally distinct molecular profiles and, thus, need to be evaluated individually for suitable targeted therapies. Although statistically nonsignificant, our data further indicate that patients with multifocal, clonally unrelated tumours may have a better outcome compared with multifocal, clonally related tumours, most probably due to the fact that metastatic intrapulmonary spread has already occurred in the latter.

About 60 yrs ago, SLAUGHTER *et al.* [18] initially noted that smokers develop multiple pre-neoplastic lesions and synchronous or metachronous tumours in the squamous epithelium of the oral cavity, a phenomenon they termed field cancerisation. This concept was later extended to the entire upper aerodigestive

tract [19] and clinicopathological criteria were developed to help identify the origin of multifocal tumours [10–14]. For NSCLC, careful evaluation of histomorphological criteria such as growth pattern in ADC is a key approach in the initial assessment of these cases [12]. However, a definitive statement of clonality may only be obtained by molecular testing, as demonstrated by GIRARD *et al.* [20] in a small series of synchronous NSCLC that were analysed by comparative genomic and mutational profiling analyses.

To distinguish clonally distinct NSCLC from intrapulmonary tumour spread means identifying patients with possible effects of a field cancerisation (*i.e.* due to carcinogen exposure or a genetic background). For the origin of multiple, synchronous, histological indistinguishable tumours, two mechanisms have been proposed: 1) a single clonal event resulting in a tumour with subsequent spread into other parts of the lungs; and 2) multiple independent tumours arising in an area as a consequence of carcinogen exposure. In the past, different molecular approaches have been utilized to address this issue. By analysing LOH and *p53* mutations in a series of 14 synchronous NSCLC cases, SHIMIZU *et al.* [21] reported a clonal relationship in 11 (79%) cases. This is in accordance with a study by WANG *et al.* [6], who observed a clonal relationship in 77% of 30 patients with multifocal lung tumours according to LOH, *p53* mutations and X-chromosome inactivation status. With a combined assessment of LOH (using a panel of 14 polymorphic microsatellite markers) and *KRAS* and *EGFR* mutations, we provided evidence that $\geq 64.1\%$ of synchronous ADCs and SQCCs have a clonal relationship.

Since smoking has been recognised as a major cause of field cancerisation in the oral cavity [18], we investigated whether this holds also true for the lower respiratory tract. Although statistically nonsignificant, we demonstrated that both ADC and SQCC patients with clonally unrelated tumours had higher numbers of pack-years compared with patients with clonally related tumours, thus supporting the association of extensive smoking and the development of a pulmonary field cancerisation.

A different prevalence of the main driving mutations in the *KRAS* and *EGFR* genes between primary NSCLC and associated lymph node or distant metastases has been reported with discordance rates of up to 33% [22–30], thus challenging the concept of a tumour-specific therapy. However, a 100% concordance was reported in a series of six NSCLCs and their corresponding brain metastases [23], possibly indicating that different metastatic sites may influence clonal selection and, thus, the rate of *EGFR* mutations. In our series, one patient with two synchronous foci of ADC was shown to have a *KRAS* mutation in one and an *EGFR* mutation in the other intrapulmonary tumour nodule. Another ADC case harboured two different *KRAS* mutations (case 10; G12C and G12S). These findings strongly argue against a clonal relationship of these tumours and demonstrate that the mutational status of *EGFR* and *KRAS* is helpful to specify the clonal relationship of synchronous ADC.

According to the present NSCLC cohort, there are no specific anatomical or morphological criteria that reliably indicate a clonal or a nonclonal origin of multiple tumours in the lungs.

Both constellations were evident within one lobe, in different lobes, and also associated with or without lymph node metastases. Therefore, for practical reasons, we recommend performing predictive molecular testing separately in all cases with synchronous tumour nodules. Alternatively, allelotyping may be performed to identify clonally related tumours, for which testing of one tumour nodule alone may be sufficient. This approach is also applicable to biopsy material of nonresectable, multifocal NSCLC.

Assessment of clonality may provide further prognostically relevant information. Patients with multifocal, clonally unrelated tumours tended to have an improved survival compared with patients with clonally related tumours. However, the low numbers of clonally unrelated tumours with different Union for International Cancer Control stages were major limitations for statistical analyses. The prognostic impact of clonality should be analysed further in large cohorts of NSCLC in order to optimise treatment decisions and patient outcome.

In conclusion, as cases of nonresectable, synchronous NSCLC with divergent mutational status of *KRAS* and *EGFR* are likely to show different responses to *EGFR*-targeted therapies, pathologists and clinicians must be aware of the need to separately analyse multifocal tumours for predictive biomarkers when histomorphological criteria alone do not allow a reliable discrimination. Naturally, besides *EGFR*, this also holds true for further, novel molecular predictors of future therapeutic targets.

STATEMENT OF INTEREST

None declared.

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