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Spatial and temporal heterogeneity of lung adenocarcinomas in tumor tissues and circulating DNA

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Lung adenocarcinoma (ADC) is the most prevalent subtype of lung cancer and represents an entity with considerable heterogeneity. Increasing evidence suggests the crucial role of intratumor heterogeneity (ITH) as a driver of tumor progression and the development of therapy resistance through the selection of molecularly altered subclones. The coexistence of distinct cellular subpopulations with differing genotypes and phenotypes poses a major challenge for molecular stratification and treatment prediction. Therefore, this thesis aimed at the examination of spatial and temporal heterogeneity of ADCs on the histological, genetic, and epigenetic level in tissue and blood samples.

The first objective was to investigate the regional distribution of respective EGFR and KRAS mutation frequencies in correlation with the tumor cell content and morphological patterns in ADCs. To this end, central tumor sections from 19 ADCs were subdivided into 467 malignant segments and 147 non-malignant segments. Histopathological evaluation at the Institute of Pathology Heidelberg showed the extensive histological ITH of ADCs with multiple growth patterns in 16 of the 19 primary tumors and revealed notable variability in the cellular fractions of neoplastic and non-neoplastic cells between the distinct patterns. Molecular analysis confirmed the omnipresence of EGFR or KRAS driver gene mutations in all tumor regions with highly variable abundances throughout the tumors and between the individual cases. The frequencies of the mutant alleles correlated significantly with the distinct histological growth patterns, but not with the tumor size. The pervasive presence of EGFR or KRAS mutations indicated their clonal origin. Thus, although a single biopsy is sufficient for the detection of driver gene mutations to derive potential therapeutic interventions, it is not representative for the entire spectrum of morphological and molecular diversity of ADCs.

Considering the strong histological and genetic ITH of ADCs, the second objective concerning spatial heterogeneity of this thesis was to gain insights into the epigenetic heterogeneity of ADC in order to find variations between the diverse histologies and lymph node metastases as well as to reconstruct the clonal tumor evolution. In this project, global DNA methylation profiling of 27 primary tumor regions, matched normal tissues, and six lymph node metastases from seven ADC cases was performed. All regions showed varying degrees of methylation changes between segments of different, but also of the same morphologies. Similarly, clonal and subclonal copy number variations were seen between spatially distinct segments of each patient. Hierarchical clustering of methylation patterns detected extensive heterogeneity within and between the individuals. Intra-tumor DNA methylation heterogeneity revealed a branched clonal evolution of ADC regions, which was driven by genomic instability. Notably, methylation profiles within tumors were not generally more similar to each other than to those from other individuals. Importantly, none of the lymph node metastases was closely related to other subclones or histologies in the same case, suggesting that the metastases had evolved early and separately from the primary tumors' growth patterns. The data illustrate that strong clonal dynamics are the basis for the extensive heterogeneity of lung ADCs. Comprehensive multiregional analysis of genomic, epigenomic, and histological variations in ADC patients may allow to identify the connections between molecular and morphological ITH of ADCs.

In addition to the spatial heterogeneity, a second aim was to investigate temporal changes of NSCLC under therapy. ADC patients with activating mutations in the EGFR gene are eligible for treatment with tyrosine kinase inhibitors (TKIs) and benefit from longer overall survival. However, the tumors

acquire resistance due to the emergence and selection of molecularly altered subclones. Thus, serial therapy monitoring is mandatory to detect the appearance of resistance and disease progression. To address this issue, a continuous non-invasive monitoring of tumors over time based on the level of mutant circulating tumor DNA (ctDNA) was initiated in order to identify molecular indicators of therapy response and tumor progression, including the occurrence of resistance mutations, e.g. EGFR T790M. Serial plasma samples (n=107) from 16 ADC patients, treated with anti-EGFR TKIs, were used to quantify EGFR and KRAS mutations in ctDNA by digital PCR. Temporal variations in mutation levels were found in almost all patients, with the earliest sign for response 26 hours after therapy onset. Low levels of mutant DNA over time were concurring with stable disease. Progressive disease and occurrence of resistance were detected up to several months prior to radiological progression. Vast numbers of mutations in ctDNA were associated with poor prognosis and short overall survival. Together, early and serial ctDNA evaluation in ADC patients provides molecular evidence of tumor burden, which can be associated with clinical disease courses. Rising ctDNA levels can be detected before tumor progression becomes clinically evident and thus might support earlier treatment decisions and improve prognoses. However, to translate serial ctDNA analysis into routine clinical diagnostics, larger patient cohorts and systematically controlled studies are required, which include standardization of pre-analytical and analytical procedures.

In order to detect mutations besides the prominent hotspots and to investigate the molecular representation of the mutational tumor profile in ctDNA, whole-exome sequencing (WES) of serum and matched tumor tissue samples from six NSCLC patients and two control sera was performed. WES identified cancer-associated mutations and variants at COSMIC annotated sites in all six patients. The results demonstrate that ctDNA is able to inform about the molecular constitution of the disease in the six advanced cancer patients with a median of 17% (range 5.2% - 56.7%) of the tumor variants to be represented in the matched serum samples. In addition, ctDNA analysis detected a MTOR mutation of potential clinical relevance, which had not been found in the primary tumor. In summary, WES of ctDNA notifies about the primary tumors' molecular alterations and can provide complementary information about the mutational patterns eventually deriving from distant tumor clones. While more patient samples are required to evaluate the informative value of WES of cfDNA, the results provide evidence for global approaches to comprehensively cover the extensive molecular tumor heterogeneity in serum and plasma of cancer patients.

Of course, further standardization of analytical procedures as well as improved understanding of the biology of cfDNA is required to translate liquid biopsies into widespread clinical routine. However, the results of this thesis illustrate the utility of ctDNA analyses for molecular characterization and minimal invasive disease monitoring, suggesting the potential of liquid biopsies to be of high value for the management of cancer patients. In conclusion, the investigations of genetic, epigenetic, and histological heterogeneity in lung cancers, and the possibility to dynamically track these alterations in the blood, can help to increase the understanding of ITH and how it contributes to the development of tumor progression, and therapy resistance.