The Role of Molecular Diagnostics in Cancer Diagnosis and Treatment

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Keywords

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Summary

The field of molecular diagnostics has improved our understanding of the pathophysiological mechanisms of malignant alteration, especially in lung cancer which remains the leading cause of cancer-related mortality worldwide. The role of the epidermal growth factor receptor (EGFR) and other molecules linked to the EGFR signaling pathway has been extensively studied, and they have become the target of new, specific therapies in lung adenocarcinomas. Similarly, the amplification of the fibroblast growth factor receptor 1 (FGFR1) gene described in squamous cell lung carcinomas has opened new possibilities for molecular targeted therapy. Next generation sequencing (NGS) methods have made possible an even more accurate detection of rare somatic mutations.

Introduction

Molecular diagnostics methods play an increasingly important role in understanding the pathogenesis of various diseases as well as in their diagnosis and treatment. Analysis of HER2 amplification status in breast cancer and KRAS mutations in colorectal cancer have already been widely accepted as a supplement to conventional histological examination. A big step forward in elucidating tumor pathogenesis was made upon identification of the epidermal growth factor receptor (EGFR) and its role in lung cancer which remains the most common cause of cancer deaths in men and the second leading cause of cancer deaths in women [1]. Molecular analysis of the EGFR, its corresponding downstream signaling cascades, and their mutations has paved the way for novel, molecular targeted cancer therapies, proving that this type of

Schlüsselwörter

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Zusammenfassung

Die molekulare Diagnostik hat unser Verständnis pathophysiologischer Mechanismen maligner Veränderungen verbessert. Dies gilt insbesondere für das Bronchialkarzinom, das weiterhin die weltweit führende Ursache der krebsassoziierten Todesfälle ist. Die Rolle des epidermalen Wachstumsfaktorrezeptors (EGFR) und seiner Signaltransduktionswege wurde ausführlich untersucht und damit Angriffsziele für neue, spezifische Therapien des Adenokarzinoms identifiziert. Ebenso wurde eine Amplifikation des Fibroblasten-Wachstumsfaktor-Rezeptor-1-Gens (FGFR1) beim Plattenepithelkarzinom der Lunge beschrieben, womit neue Möglichkeiten für die molekular gerichtete Therapie eröffnet worden sind. Eine präzisere Detektion somatischer Mutationen wird durch die Next-Generation-Sequenzierung ermöglicht.

analysis performed on histological material is becoming increasingly important in cancer diagnostics and patient stratification for therapy.

EGFR in Lung Adenocarcinoma

The EGFR is a transmembrane tyrosine kinase that belongs to the ErbB family of receptors. It possesses an extracellular, transmembrane as well as intracellular domain, so that binding of the specific ligands, including the epidermal growth factor (EGF), transforming growth factor α (TGF α), and the neuregulins, induces a series of intracellular signaling cascades [2, 3]. Binding of the ligand to the extracellular domain causes dimerization with other receptor molecules. Both homodimerization with another EGFR receptor and

Fig. 1. Signaling pathway mediated by EGFR (simplified).

heterodimerization with other members of the ErbB family are possible. As a result, the receptor undergoes a conformational change with reorientation of the intracellular domain in its activated kinase configuration [3]. The activated EGFR kinase catalyzes phosphorylation of tyrosine residues of other intracellular proteins as well as autophosphorylation of its own C-terminal domain. The phosphorylated EGFR undergoes interactions with several proteins that initiate other signal transduction cascades, among which MAPK, STAT3, and Akt signaling pathways are known for their role in lung cancer pathophysiology [4]. A simplified schematic illustration of the EGFR signaling pathway is shown in figure 1. EGFR is normally expressed in tissues of epithelial, mesenchymal, and neuronal origin [5] and plays an important role in cell differentiation and proliferation [6]. However, it is also involved in cell survival, growth, and metastatic spread [7, 8] of various tumors, including head and neck cancer, breast cancer, lung cancer, colorectal cancer, kidney cancer, prostate cancer, ovarian cancer, and glioblastoma multiforme [9]. EGFR overexpression has also been correlated with advanced stages of disease, poor prognosis [10–12], and resistance to radiation, chemotherapy or hormonal therapy [13, 14].

Therapeutic Possibilities

Due to its role in cancer development and progression, EGFR has become a potential target for cancer therapy, and several drugs directed towards it have already been developed. These can be categorized into 2 groups: monoclonal antibody inhibitors and low molecular weight tyrosine kinase inhibitors [15, 16]. Anti-EGFR monoclonal antibodies (cetuximab, matuzumab) bind to the extracellular part of the EGFR. By competitively blocking the ligand-binding domain, they inhibit the ligand-induced activation of the receptor [16, 17]. Low molecular weight EGFR tyrosine kinase inhibitors (gefitinib, erlotinib) reversibly bind to the ATP binding site at the intracellular domain of the EGFR and in-

hibit its autophosphorylation, thus blocking the downstream intracellular signal transduction pathways [18, 19].

The first biomarker used to test the efficacy of anti-EGFR therapy in cancer treatment was the semiquantitative analysis of EGFR expression by immunohistochemistry. However, most of the studies could not correlate EGFR expression to the clinical efficacy of EGFR antagonists [20, 21]. An increased EGFR gene copy number was demonstrated in non-small cell lung cancer (NSCLC), squamous cell carcinoma of the floor of the mouth, and colorectal carcinoma by fluorescence in situ hybridization (FISH) analysis. One of the first reports that correlated the EGFR gene copy number with cetuximab/panitumumab treatment response referred to a cohort of 31 patients with metastatic colorectal cancer [22]. However, the INTEREST (IRESSA Non-small cell lung cancer Trial Evaluating Response and Survival against Taxotere) study could not correlate the EGFR gene copy number with tumor progression or overall survival of patients treated with gefitinib [23].

Sequencing of the EGFR gene and identification of somatic mutations in its tyrosine kinase domain have improved the understanding of these mechanisms. Activating mutations in the EGFR gene have been reported to be associated with hypersensitivity to tyrosine kinase inhibitors in patients with advanced NSCLC [24].

Mutations and Resistance Mechanisms

EGFR mutations mostly affect exons 18–21 of the EGFR gene. In-frame deletions in exon 19 and a point mutation in exon 21 that leads to substitution at codon 858 (L858R) are most commonly found. A recent study has defined exon 19 deletions or L858R mutations as best predictive factors for time to treatment failure in chemotherapy-naïve NSCLC patients on gefitinib [25]. Sequence analysis of exons 18 and 21 is shown in figure 2.

Another study involving a larger number of patients with lung cancer [26] has confirmed the importance of screening for EGFR mutations before making decisions about treatment, and has helped to establish molecular analysis of lung adenocarcinoma as a routine in determining the mutation status of a tumor and predicting the response to treatment with EGFR tyrosine kinase inhibitors.

In addition to EGFR mutations, pathogenesis of lung cancer may also involve mutations in other genes of the EGFR signaling pathway. 2 main intracellular pathways activated by EGFR are the RAS/MAPK and PIK3CA/AKT pathway [27]. Mutations in the KRAS gene can be found in 15–36% of the patients with NSCLC and may lead to an EGFR-independent activation of MAPK [28]. These mutations almost never occur simultaneously with EGFR mutations and are associated with resistance to EGFR tyrosine kinase inhibitors [29]. Similarly, mutations in the BRAF gene activate the EGFR signaling

Fig. 2. Sequence analysis of exons 18 and 21: **A** G719A mutation in the EGFR exon 18 results in a substitution of glycine at position 719 for an alanine; **B** similar mutations at different positions in exon 21.

pathway and lead to the development of lung carcinomas with predominantly papillary or bronchioloalveolar growth pattern [30]. Mutations in PIK3CA occur in less than 5% of patients with lung cancers [31] but may also lead to lower response to EGFR tyrosine kinase inhibitors [32]. Although less commonly, other EGFR pathway genes, including HER2 and HER4, may also be affected by somatic mutations [33].

Another oncogene has been identified in a subset of NSCLC patients, which is the result of fusion of the echinoderm microtubule-associated protein-like 4 gene (EML4) and the anaplastic lymphoma kinase gene (ALK) [34]. Patients who harbor this mutation are more likely to be young men who have never smoked [35]. Histologically, EML4-ALK-positive tumors show predominantly a signet ring cell [35] or acinary [36] pattern. The EML4-ALK fusion gene encodes a pathologically hyperactive tyrosine kinase, and these tumors usually lack both EGFR and KRAS mutations [36]. Thus, the patients do not show response to EGFR tyrosine kinase inhibitors and are candidates for treatment with an ALK inhibitor therapy and should be screened for the translocation by FISH [35]. A c-Met/ALK-inhibitor is currently being tested in a phase III clinical trial [37].

Up to 50% of patients with NSCLC, who initially responded to gefitinib/erlotinib therapy, develop a secondary resistance to treatment, which is mostly due to a substitution of methionine for threonine at codon 790 (T790M) in exon 20 [38, 39], leading to a conformational change at the ATP-binding site of the tyrosine kinase that makes binding of the tyrosine kinase inhibitors impossible [40]. This mutation was reported to be detectable in some patients before treatment and correlated with lower survival rates [41]. Other resistance mechanisms include loss of the PTEN tumor suppressor gene with consequent activation of EGFR and Akt [42], as well as amplification of the MET proto-oncogene that leads to HER3-dependent PIK3CA activation [43].

FGFR in Squamous Cell Lung Carcinoma

In contrast to adenocarcinoma, no specifically targeted therapies have been reported in squamous cell lung carcinoma. However, a focal amplification involving another protein

Fig. 3. Lung biopsy. Lung tissue infiltration by an adenocarcinoma.

important for the regulation of cell growth and maturation – fibroblast growth factor receptor 1 (FGFR1) – has recently been reported. The studies conducted show that frequent and focal FGFR1 amplification is associated with growth and survival in squamous cell lung cancer [44, 45]. This suggests FGFR1 as a potential target for novel anticancer drugs. Clinical trials with FGFR1 inhibitors are already in progress, including a phase I study that has recently been initiated at the Center for Integrated Oncology (CIO) Cologne Bonn [46].

Current Analyses and Potential Problems

Currently, most molecular analyses are performed as sequential single assays either based on high resolution melting curve analysis for genes where hotspot mutations are known, i.e. BRAF, KRAS, PIK3CA, or direct standard Sanger sequencing of relevant exons in for example EGFR. At the CIO Cologne Bonn, these analyses are routinely used as part of extended predictive diagnostics. They are all performed on formalin-fixed paraffin-embedded tissues (FFPE) and require

a relatively high tumor content to allow for mutation detection. Figure 3 shows a representative small lung biopsy infiltrated with an adenocarcinoma used for routine diagnostics. Since the analyses are performed sequentially, results can take up to 3 weeks, which with limited hospital stays may present a significant bottleneck. It is therefore recommended that samples for DNA extraction be prepared in parallel with the conventional HE slides, especially in cases where primary diagnostics are done outside the centre for molecular diagnostics. In summary, there is an urgent need to firstly speed up the molecular pathologic analysis and also to reliably detect low frequency mutations in samples with limited tumor content. The current challenge is to implement next generation parallel sequencing approaches to address both these issues.

New Perspectives by Next Generation Sequencing Technologies

Since the novel and individualized therapeutic approaches of NSCLC depend on the detection of a wide and increasing panel of somatic mutations in signal transducers, fast and highly sensitive molecular diagnostic tools are required. In the last 5 years, next generation sequencing (NGS) methods have fundamentally changed genomic analyses, providing a more accurate and sensitive detection technology of rare somatic mutations in the tumor [47, 48]. Though the NGS chemistries are varied reaching from pyrosequencing, to polymerase-based primer extension and iterative oligonucleotide ligation, the strategy in common is the read through many different DNA templates in parallel but in separated flow cells. Thus, millions of sequencing reads of the DNA templates are generated in parallel, gathering information of up to 200 Gb, while the read lengths for each DNA template are very short (35–500 bp) compared to the traditional Sanger sequencing method [48]. This principle of highly parallel sequencing of single or clonally amplified DNA molecules, each analyzed in a single separate flow cell, enables the analysis of a DNA population with low numbers of mutated DNA molecules against a large background of wild-type DNA.

The parallel sequencing techniques can be applied to a panel of certain relevant genes that are prone to harbor can-

Fig. 4. Data of parallel sequencing showing numerous reads of exon 19 of the EGFR. Next generation sequencing (NGS) data, generated by pyrosequencing and read by the GS Junior (454 Life Sciences/Roche, Branford, CT, USA), were analyzed with Amplicon Variant software (454 Life Sciences/Roche). **A** The deletion in exon 19 of the EGFR gene is shadowed in the pyrosequencing flow diagram. **B** Multiple reads ($n > 600$) of the exon 19 locus revealed in approximately 50% of the sequences a deletion in exon 19 of the EGFR gene.

cer-specific point mutations or deletions (fig. 4), but also to a comprehensive assortment of signal transducers involved in signaling of tumor cells, or even to the entire transcriptome, exome, or genome of the tumor cells. These perspectives will take center stage in the identification of novel genetic cancer markers and in future molecular diagnostics supporting an improved personal therapy of the patient [49].

Disclosure Statement

The authors declare no conflicts of interest.

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