Personalized Medicine Review Series *J. Cell. Mol. Med. Vol 14, No 4, 2010 pp. 805-817*

Guest Editors: R. Y. Osamura & A. M. Gown

Oncologist's/haematologist's view on the roles of pathologists for molecular targeted cancer therapy

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Received: August 8, 2009; Accepted: February 9, 2010

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Abstract

In the past two decades there has been a tremendous increase in the understanding of the molecular basis of human malignancies. In a variety of neoplasms, specific molecular markers became part of disease classifications and are now routinely used to define specific entities. Molecular analyses discriminate prognostic groups, guide differential treatment strategies and identify targets for molecular defined cancer therapy. A battery of new drugs has been developed to specifically inhibit oncogenic pathways. For an increasing number of solid and haematological malignancies, the availability of molecular targeted drugs has fundamentally changed treatment algorithms. However, the diagnostic, prognostic and therapeutic impact of selected molecular markers is still limited in many cases. After all, the success of a molecular targeted therapy is clearly determined by the significance of the targeted structure for the biology of cancer and the ability of the malignant cell to evade specific inhibition.

Keywords: biomarkers • targeted therapy • molecular targets

Introduction

Targeted therapies have been already used successfully for decades in various malignancies. Examples include tamoxifen as an anti-oestrogenic therapy in oestrogen receptor positive breast cancer [1, 2] or radioiodine in iodine-avid thyroid cancer [3]. More recently, the anti-CD20 monoclonal antibody rituximab [4] and the radioactive-labelled anti-CD20 antibodies ibritumomab tiuxetan [5] and tositumomab [6] have emerged as targeted therapy in $CD20^+$ non-Hodgkin lymphoma.

However, very few malignancies are actually caused and driven by a single or limited number of oncogenic events that combine (bio)marker and target characteristics. One of the rare examples is chronic myelogenous leukaemia (CML), where the t(9;22)(q34;q11) balanced reciprocal translocation generates the Bcr-Abl oncogene that encodes a constitutively active tyrosine kinase. The development of the Abl tyrosine kinase inhibitors (TKI) imatinib mesylate was facilitated by progress in structure-based drug development and resulted in a highly efficient, well-tolerated therapy that has displaced more aggressive treatment modalities from first line therapy [7]. However, most malignant diseases are driven by a highly complex and plastic survival network that

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doi:10.1111/j.1582-4934.2010.01032.x

evolves during tumorigenesis. This oncogenic network can rapidly change and adapt during anticancer therapies and progression [8–11].

Recently, a plethora of potentially suitable drugs that target surface, cytoplasmic or nuclear structures of tumour cells have been developed, that inhibit pathway activation or that hinder tumour–stroma interactions. Therefore, there is an increasing requirement for informative biomarkers that can be used to identify promising applications for these new compounds and to allow discrimination of prognostic groups. The identification of prognostic and therapeutic biomarkers, that allow identification of suitable patients and disease monitoring during therapy, represents a major diagnostic challenge for therapies that use molecular targeted drugs.

Techniques used for diagnosis and monitoring of malignant diseases treated with targeted therapies

Sophisticated methods are available today for molecular diagnostics and biomarker identification. Most of these analytical techniques are in the hands of pathologists. High throughput gene expression analysis allows the time efficient acquisition of the whole transcriptome in a given sample [12, 13]. High throughput DNA sequencing complements this technique in molecular diagnosis and monitoring [14, 15]. Pharmacogenomics correlate gene expression or single nucleotide polymorphisms with drug efficacy or toxicity [16]. Finally proteomic analysis and most recently metabolomic analysis are emerging to extent the technical spectrum on the protein and metabolite level. Although not used in routine diagnostics thus far both proteomics and metabolomics have been applied in cancer cell lines and primary cancer cells. Compared with gene expression studies and gene sequencing, proteomics and metabolomics provide the intriguing opportunity to assess changes downstream of transcription and thus might more appropriately reflect phenotype and function [17–19].

Chronic myelogenous leukaemia: the poster child of targeted therapy

CML has become the paradigm for targeted cancer treatment. The introduction of imatinib has led to a dramatic improvement of prognosis in chronic phase patients when compared to conventional chemo-/immunotherapy. Imatinib has become the gold standard in the treatment of CML with excellent and durable responses and minimal side effects. The use of small molecule kinase inhibitors has been extended to other malignant entities, and thereby redefined the management of cancer in general.

Bcr-Abl as target for therapeutic kinase inhibition

Protein tyrosine kinases are enzymes that transfer phosphate groups from ATP to substrate proteins, thereby governing cellular processes such as growth and differentiation. Tight regulation of tyrosine kinases is indispensable and, if not maintained, deregulated kinase activity can lead to transformation and initiation of malignancy. The Philadelphia chromosome, first described as a shortened chromosome 22 [20], results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [21], and is present in approximately 95% of CML patients and up to 20% of adult acute lymphoblastic leukaemia (ALL) [22, 23]. The Philadelphia translocation gives rise to the oncogenic Bcr-Abl fusion protein that is characterized by a constitutively active tyrosine kinase. Bcr-Abl is sufficient to cause CML in mice [24], and its transforming capacity strictly depends on tyrosine kinase activity [25]. This makes Bcr-Abl an attractive target for therapeutic intervention in CML and Ph + ALL.

Preclinical and clinical development of the TKI imatinib

The 2-phenylaminopyrimidine class of small-molecule kinase inhibitors was identified using a high throughput screen of compound libraries at Ciba-Geigy (now Novartis, Basel, Switzerland) [26]. The phenylaminopyrimidine CGP57148B (Imatinib-mesylate, hereafter imatinib), a derivative of the initial lead compound, was found to inhibit autophosphorylation of four kinases: The receptor tyrosine kinase platelet-derived growth factor receptor (PDGF-R) α and β, the receptor tyrosine kinase cKit and the protein tyrosine kinase Abl, including its close homologue Arg [27, 28]. Imatinib has been demonstrated to specifically inhibit oncogenic derivatives of these kinases including Bcr-Abl [29, 30] and cKit harbouring oncogenic mutations [31]. Preclinical studies demonstrated activity in Bcr-Abl$^+$ cell lines and in animal models [26, 29, 32]. Based on these observations, clinical trials in Bcr-Abl$^+$ CML were initiated in 1998.

Phase 2 clinical trials demonstrated activity of imatinib in chronic phase as well as in accelerated phase (AP) and blast crisis (BC) CML [33–35] and lead to the approval of imatinib for the treatment of CML in 2002. Activity was reported in patients with chronic phase CML and IFN resistance or intolerance [36]. A phase 3 clinical trial (IRIS trial) documented the superiority of imatinib over IFN in combination with low-dose cytarabine in patients with newly diagnosed, untreated chronic phase CML with respect to haematological, cytogenetic and molecular responses [37, 38], and also with respect to overall survival [39, 40]. After 7 years, 60% of patients randomized to receive imatinib were still on study medication, and the overall survival rate for patients randomized to imatinib (intention to treat) was 86% or 94% when only CML-related deaths were considered [41]. Imatinib side effects were mainly considered as mild or moderate [37, 42]. These results were reproduced outside the setting of a clinical trial [43] and established imatinib 400 mg daily as standard treatment for patients with CML in chronic phase [44, 45].

While imatinib leads to sustained responses in the majority of chronic phase CML cases, responses in advanced phase CML usually are short lived [34, 35]. Therefore, patients in BC should proceed to allogeneic haematopoietic stem cell transplantation as soon as a haematological response has been achieved [44]. Patients in AP should be closely monitored, and in case of a loss of haematological or cytogenetic response (CyR), should be submitted to stem cell transplantation.

CML: molecular diagnostics guide treatment

According to the techniques used for monitoring, three levels of response can be discriminated [44, 46]. With decreasing leukemic burden, the primary finding will be the normalization of blood cell counts (haematological response). Later on, the decrease of Philadelphia-positive metaphases in the bone marrow indicates CyR. Molecular response is reflected by a decrease of Bcr-Abl transcripts in peripheral blood or bone marrow using quantitative real-time PCR (qRT-PCR).

Haematological and CyR to first-line imatinib at 3, 6 and 12 months in patients with chronic phase CML determines progression-free and overall survival. Patients who are continued with imatinib despite a lack of CyR face the risk of progression to accelerated and blast crisis. In contrast, achieving a complete CyR is associated with excellent progression-free survival, provided that imatinib is continued without dose reduction or interruptions [37, 47]. Thus, regular monitoring of imatinib treatment in CML is indispensable to confirm adequate response and to identify patients with suboptimal response or treatment failure early enough to make appropriate treatment changes [44, 47].

A failure to achieve haematological response and loss of a previously achieved haematological or CyR are rare events in chronic phase CML [37, 47]. However, primary cytogenetic failures are more prevalent [42, 47]. In contrast, in advanced phase CML primary haematological failure occurs more frequently, and after 4 years, resistance to imatinib had emerged in 45–70% of cases in AP, and 90% in BC, respectively [48–51].

Molecular mechanisms that frequently cause clinical resistance to imatinib include Bcr-Abl gene amplification and protein overexpression [52, 53], clonal cytogenetic evolution [53–55] and most importantly, mutations of the *Bcr-Abl* kinase domain that lead to structural changes so that imatinib is no longer able to displace ATP [52, 53, 56–59]. Importantly, not only treatment failure itself but also molecular mechanisms leading to resistance can be identified by molecular diagnostic procedures that are routinely performed during treatment monitoring: Conventional cytogenetic analysis (clonal cytogenetic evolution), fluorescence *in situ* hybridization (FISH; Bcr-Abl gene amplification), denaturing high-performance liquid chromatography (DHPLC; screening for *Bcr-Abl* gene mutations) and sequencing of the *Bcr-Abl* kinase domain.

The finding of clinical resistance to imatinib triggered the development of novel Abl kinase inhibitors. Preclinical models revealed a higher inhibitory activity of these drugs against wild-type Bcr-Abl in cell lines and animal models, and also demonstrated activity of these novel compounds against many of the known imatinib resistant Bcr-Abl exchanges. Examples include nilotinib (AMN107) [60], and dasatinib (BMS354825) [61]. Both nilotinib and dasatinib have been demonstrated to induce haematological responses in imatinib intolerant and resistant CML [62–66] and have been approved for the treatment of imatinib resistant or intolerant CML.

In the treatment of CML with imatinib, molecular diagnostics constitute an integral part of the routine monitoring. Results of cytogenetic analysis and qRT-PCR indicate suboptimal response or treatment failure and should trigger *Bcr-Abl* mutation analysis. The presence of an individual resistance mutation is one of the factors that determine the choice of the appropriate further treatment (Fig. 1).

Lessons learned from CML targeted therapy: c-Kit, PDGFR and EGFR dependent tumours

Mutations conferring clinical resistance to therapeutically used kinase inhibitors were also identified in several other target kinases in various malignant diseases. Imatinib resistance mutations were identified in *FIP1L1-PDGFR*α in patients with hypereosinophilic syndrome [67, 68], and in cKit in patients with gastrointestinal stromal tumours (GIST) [69, 70]. In addition, a resistance mutation in the kinase domain of *FLT3-ITD* in a patient with acute myeloid leukaemia treated with the kinase inhibitor PKC412 has been described [71]. Similarly, in patients with non-small cell lung cancer (NSCLC) treated with the kinase inhibitor gefitinib, an exchange of threonine at position 790 to methionine in the *epidermal growth factor receptor* (*EGFR*) was reported [72, 73]. Interestingly, this mutation together with the imatinib resistant mutations *cKit*/T670I and *FIP1L1-PDFGR*α/T674I are homologous to the position T315 in the *Abl* kinase domain. Thus, mutations in kinase domains seem to be a general mechanism of resistance against the class of TKIs and clearly demonstrate that TKIs used to treat these diseases hit critical targets.

While cytogenetics and PCR are routinely used to establish the diagnosis and to monitor residual disease in leukaemia, the application of molecular diagnostic tools in solid tumours is heretofore routinely used only in a limited number of specific entities. In GIST, activating mutations of *cKit* or *PDGFR*α can be identified in 85 per cent of the cases and treatment with imatinib, which inhibits both cKit and PDGFRα induces responses in the majority of cases [74]. Like in CML, resistance to imatinib in GIST is associated with mutations in the *cKit* or *PDGFR*α kinase domain, and second line TKI treatment with sunitinib can be active after imatinib failure [75]. Therefore, GIST tumours represent an example of a solid tumour entity uniformly addicted to a specific oncogenic kinase (mostly cKit). Molecular diagnostics in GIST can be used for discrimination to other forms of sarcoma (cKit immunohistocehmistry), but also adds valuable prognostic information, as the *cKit* genotype determines response to imatinib [76]. Similar to GIST in which the survival of the tumour cells strictly depends on a growth factor receptor, other solid tumours with activating mutations in growth factor receptors have been identified. 5–10% of NSCLC patients harbour mutations in the *EGFR* or *METR* and show excellent responses to

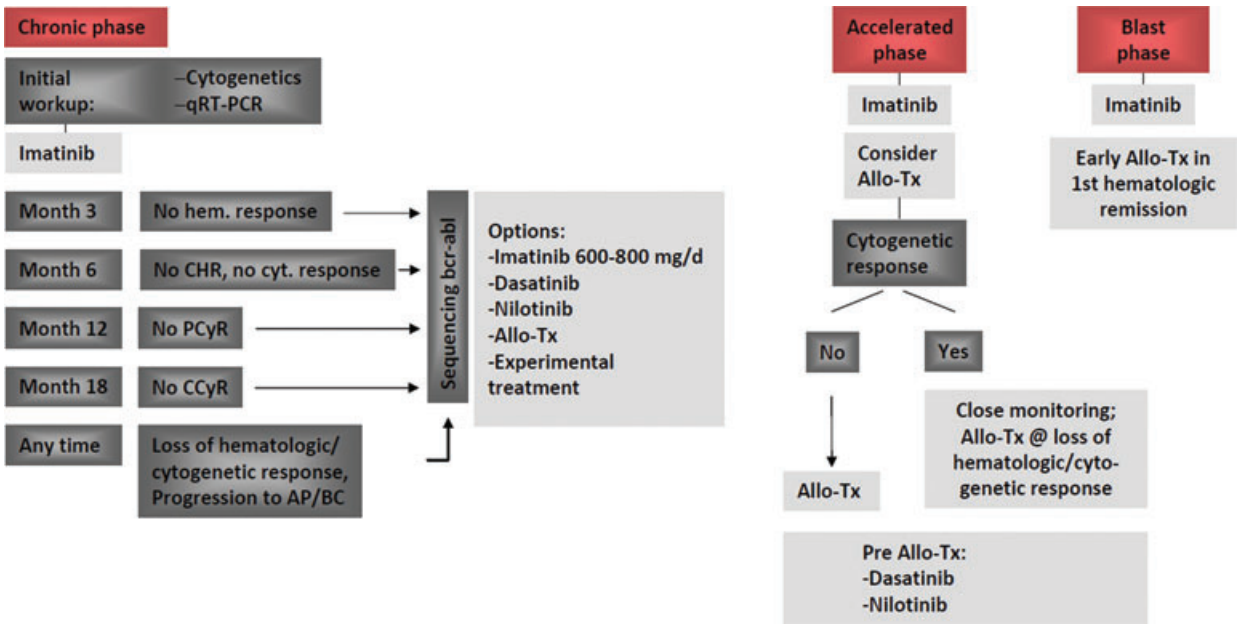

Fig. 1 Treatment algorithm in Bcr-Abl^{+} CML. Abbreviations: qRT-PCR, quantitative real-time PCR; CHR, complete haematological response; PCyR, partial cytogentic response; CCyR, complete CyR; AP, accelerated phase; BC, blast phase; Allo-Tx, allogeneic stem cell transplantation.

EGFR targeted therapy. In addition, there are a growing number of solid tumours which show amplification of the *EGFR*. In NSCLC, head and neck and colorectal cancer (CRC) EGFR is a target for already approved and effective molecular therapies.

Treating cancer with EGFR targeting therapy

Cancer is a disease of deregulated cell proliferation and impaired apoptosis [8, 9]. The capacity for autonomous and deregulated cell growth as well as inappropriate execution of cell death is often mediated through abnormal expression of growth factor receptors and the constitutive activation of their downstream signalling pathways leading to increased proliferation and survival. Many epithelial cancers are characterized by functional activation of members of the EGFR family [77–79]. EGFR is a transmembrane protein encoded by 28 exons on chromosome 7p12. It is a receptor tyrosine kinase (RTK) and belongs to the Erb family that consists of four closely related members: EGFR (ErbB1), HER-2/neu (ErbB2), Her-3 (ErbB3) and Her-4 (ErbB4). These transmembrane proteins feature an extracellular ligand-binding domain, a membrane-spanning and an intracellular domain. The major signalling pathways activated by EGFR are the RAS-RAF-MAP kinase pathway, which is mainly involved in proliferation, and the PI3K-PTEN-AKT pathway, which is mainly involved in survival [80]. EGFR seems to play an important role in tumorigenesis, since in comparison to normal control tissues, the *EGFR* gene is frequently found mutated or amplified in cancer. Furthermore, enhanced ligand expression may contribute to activation of EGFR signalling in human cancer [78, 79, 81, 82]. Targeting EGFR mediated cell proliferation and survival is therefore an attractive approach in various solid tumours. The initiation of a growth and survival signalling cascade requires receptor dimerization upon ligand binding, which subsequently leads to phosphorylation of tyrosine kinases and downstream signalling mediators [78, 83, 84]. One signalling step may be the nuclear localization of EGFR [85]. The monoclonal antibody C225 (cetuximab) was identified as a putative therapeutic as it binds the EGFR receptor and blocks subsequently phosphorylation and activation. In a xenotransplant model cetuximab resulted in suppressed growth of human cancer cells [86]. The currently available drugs that target either the ligand binding extracellular domain (monoclonal antibodies) or the kinase domain (TKI) all have substantial side effects [87, 88]. Since only a subgroup of patients treated with EGFR antagonists gain a clinical benefit, there is a pressing need to more accurately select these patients. Several studies now suggest that clinical, pathological and genetic markers help to identify patients with an expected benefit.

EGFR mutations in non-small cell lung cancer: molecular characteristics outweigh clinical characteristics

Lung cancer is the leading cause of cancer-related death in the world. About 85% of lung cancer patients have NSCLC and the majority presents with advanced disease that cannot be cured by

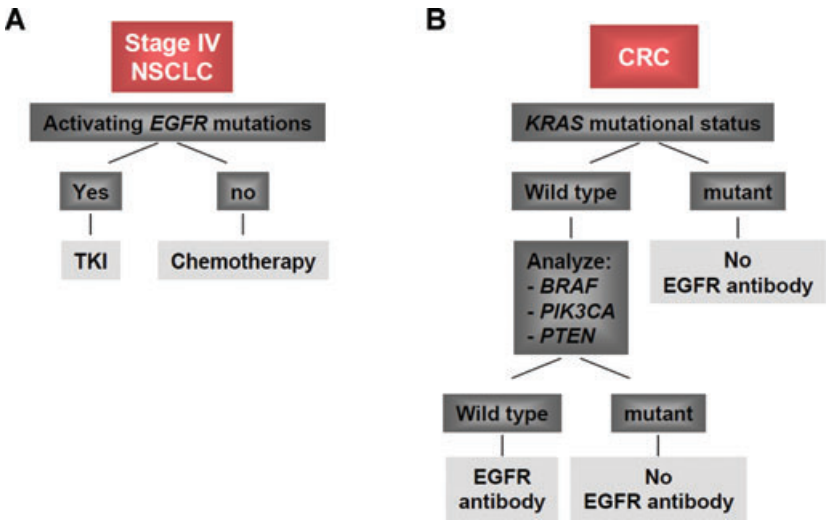

Fig. 2 EGFR targeting in solid tumours: current/possible future implication of molecular assessment. (**A**) EGFR TKI in NSCLC. (**B**) EGFR antibody treatment in CRC. Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

a surgical approach [89]. The 1-year survival using platinum-based doublet chemotherapy, which represents the current standard of care for patients with advanced disease in good performance status, is approximately 35–40% [90]. EGFR is frequently overexpressed in NSCLC and thus constitutes a promising target for therapy [78, 91]. Several mechanisms have been suggested that lead to hyperactivation of EGFR and its downstream effectors in NSCLC: overexpression of the receptor, *e.g.* by gene amplification or increased expression of ligand, or mutations of the receptor that lead to constitutive tyrosine kinase activity in the absence of ligand binding [78, 92]. Targeting the latter by using small molecule TKI (erlotinib and gefitinib) has shown promising results in clinical trials and led to the approval of TKI for NSCLC. Additional analyses established the presence of several clinical (female, adenocarcinoma including bronchioloalveolar carcinoma, never smokers) and molecular markers that are useful in predicting which patients are most likely to benefit from TKI treatment. Many objective responders had *EGFR* mutations in exon 19 or 21, which in turn have been shown to be associated with the above mentioned clinical characteristics of TKI responders [93–96]. In addition to its utility in guiding second-line therapy, this information may now permit the use of TKI for the initial management of carefully selected patients with advanced NSCLC. This approach was initially tested in a non-randomized approach in patients whose tumours harboured mutations in *EGFR* exons 18 to 21. The response rate to gefitinib monotherapy in these genetically preselected patients was 55% and the median progression free survival was 9.2 months with good tolerability [97]. Recently presented data from a randomized phase III trial that compared first line gefitinib *versus* carboplatin–paclitaxel therapy in clinically selected patients (Asians, non- or ex-smokers, adenocarcinoma, performance status of 0–2) now provides evidence that TKI treatment improves the overall response rate and reduces the relative risk of disease progression compared to chemotherapy. The superiority (RR and PFS) of TKI treatment was striking in the group of patients with *EGFR* mutations, while mutation-negative patients had a significantly higher RR in the chemotherapy arm [98]. This randomized study thus clearly provides evidence that molecular characteristics outweigh clinical characteristics and that gefitinib should be the current treatment of choice for the identified subpopulation. It has just recently led to approval of gefitinib by the European Medicines Agency as first line treatment for NSCLC that harbour activating *EGFR* mutations. More evidence for such an approach comes from a randomized phase II trial in Caucasian patients that compared erlotinib *versus* erlotinib plus chemotherapy [99].

Hence, increasing evidence supports the idea of a 'personalized' therapy for advanced NSCLC utilizing the *EGFR* mutation status as predictive biomarker before treatment initiation, and *EGFR* mutation analysis is thus required on a routine basis in pathology practice (Fig. 2A).

EGFR, EGFRvIII and other markers in head and neck cancer

Squamous cell carcinoma of the head and neck (SCCHN) is characterized by high expression of EGFR in more than 90% of tumours relative to normal control tissue, and elevated expression correlates with poor disease control and metastasis [100, 101]. This overexpression is often caused by gene amplification [102]. Furthermore, overexpression of two of its ligands, EGF and transforming growth factor-α, has been linked to a poor prognosis [103]. There is plenty of evidence that EGFR overexpression and enhanced activity of EGFR-mediated signalling is an important step in the progression of this cancer, but further events have been identified leading to the alteration of various molecular pathways that contribute to progression from premalignant lesions to invasive localized disease and to metastasis [104–107]. It has been shown that in SCCHN EGFR inhibition and conventional cytotoxic therapy collaborate in tumour control [108]. A phase III clinical trial comparing cisplatin plus cetuximab *versus* cisplatin plus placebo revealed a significant improvement in the rate of objective

responses (26% *versus* 10%), indicating at least an additive effect for the combination treatment [109]. Recently the addition of cetuximab to the standard first-line regimen cisplatin/5-fluorouracil [110] not only increased the rate of objective responses but also improved progression-free and overall survival in patients with recurrent or metastatic SCCHN [111]. These results led to the approval of cetuximab for first-line combination treatment of recurrent/metastatic SCCHN. Whether patients expressing the extracellular domain deletion mutant EGFRvIII (~42% of all patients [112]) also benefit from EGFR inhibition needs to be tested in clinical trials. Several studies are on the way examining EGFR TKI in various clinical settings in SCCHN but no drug has been approved yet. Preliminary data suggest modest activity [104, 105].

Despite the non-disputable improvement in the treatment of metastatic/ recurrent SCCHN, advancement towards a more individual approach that considers the patient's specific tumour characteristics is limited. The molecular pathogenesis of SCCHN is well established [104–107] and a number of additional pre-treatment prognostic factors (including p53 status, human papilloma virus status, expression of cell cycle regulators and anti-apoptotic proteins) have been identified that predict disease behaviour and may thus allow to choose treatment [113]. However, the definition of a patient subgroup based on biological parameters has yet been more robust than molecular assessment, and selection by response to chemotherapy [114] might currently be more reliable than genetic assessment (reviewed in [115]. Molecular profiling based on mRNA expression and analysis of genomic changes by comparative genomic hybridization arrays might nonetheless identify patients with dismal prognosis [116–118] that should be included in experimental protocols. Unfortunately, except for human papilloma virus status [114], these studies have not yet identified predictors that allow individualized treatment. Moreover, *EGFR* mutations that predict particular sensitivity to EGFR TKI [96] have not been found in SCCHN [102, 119].

SCCHN provides the advantage of easily accessible tumour tissue. This disease is therefore well suited for clinical trials in which treatment responses, especially to molecular targeted drugs, could easily be correlated to changes in signalling pathways and effects on downstream targets. The feasibility of this approach was recently demonstrated in a study that tested intralesional application of EGFR antisense DNA [120].

EGFR and KRAS, BRAF and PIK3CA mutations in colorectal cancer

Patients with metastatic colorectal cancer (mCRC) have a 5-year survival rate of less than 10% [121]. The addition of antibody therapy targeting vascular endothelial growth factor (bevacizumab) has significantly improved PFS and OS [122], presumably by inhibiting tumour angiogenesis [123]. Overexpression of EGFR has long been associated with a poor prognosis in CRC [124]. The inhibition of EGFR signalling by the monoclonal antibodies cetuximab or panitumomab has shown activity as a monotherapy and can overcome resistance to standard cytotoxic drugs [121, 125, 126]. Analysis of EGFR expression by immunohistochemistry is not predictive for response to EGFR antibody treatment and patients without EGFR expression as assessed by IHC might respond to cetuximab-based therapy [127]. Assessment of *EGFR* copy number by FISH seems to predict for response [128].

Interestingly, recent analyses of the RAS-RAF-MAP kinase pathway have shown that mutations downstream of EGFR can bypass EGFR dependence and are actually more informative than EGFR expression data. The presence of mutated *KRAS* alleles has been demonstrated to be an independent marker for resistance to the anti-EGFR monoclonal antibodies cetuximab and panitumomab [129–132]. Since less than half of the non-responders to these EGFR antibodies display mutant *KRAS* [129, 131–133] other signalling molecules caught attention as putative mediators of EGFR independence. A recent retrospective analysis revealed that the mutant *BRAF* V600E allele impairs the therapeutic effect of both approved anti-EGFR antibodies in tumours that display wild-type *KRAS*, and in fact none of the *BRAF* V600E-mutant patients responded to antibody treatment [134]. Whether the proposed combinatorial treatment with the BRAF inhibitory TKI sorafenib [135] restores sensitivity to EGFR inhibition also in a clinical setting remains to be demonstrated (Fig. 2B).

The second major signalling pathway activated by EGFR is the PI3K-PTEN-AKT pathway. *PIK3CA* encodes for a kinase that mediates EGFR signalling and is kept in check by the tumour suppressor PTEN, which is frequently mutated or deleted in human cancer [80, 136]. The *PIK3CA* gene is mutated in ~20% of CRC patients [137] and *PTEN* oncogenic mutations in CRC have been shown to be associated with clinical resistance to panitumomab and cetuximab even in the presence of wild-type *KRAS*. The authors suggest that a combinatorial molecular assessment of the PTEN-PIK3CA and KRAS pathways may identify the majority of patients unlikely to respond to anti-EGFR antibody treatment [138] (Fig. 2B). Although these retrospective analyses are of potential great importance for the clinical management of CRC patients a prospective evaluation is urgently needed before implementing such molecular assessments in routine clinical decision-making.

Taken together, analysis of the *KRAS* mutational status in metastatic CRC has become a standard approach to identify patients who will not benefit from EGFR antibody treatment. It is likely that analysis of additional predictive genetic biomarkers that bypass EGFR blockade will become a routine approach in the near future.

Diagnostic use of gene expression analysis: carcinoma of unknown primary

Despite advances in immunohistochemical analysis of tumour biopsies [139] and modern imaging techniques including 2-deoxy-glucose positron emission tomography [140] a small but

significant percentage of patients still remains with the diagnosis of carcinoma of unknown primary (CUP) [141–143]. For best effective treatment it is desirable that the origin of the tumour is identified. Molecular profiling for carcinoma of unknown primary has been shown to provide a method to identify the tissue origin in the majority of patients. One recent publication applied a combination of immunohistochemical assessment and gene expression analysis using formalin-fixed, paraffin-embedded (FFPE) samples from 84 patients with adenocarcinoma of known origin and 38 patient samples with adenocarcinoma of unknown primary origin (ACUP). An extensive immunohistochemical panel allowed classification of ACUP in 42% of the cases. Gene expression-based profiling (GEP) was superior allowing classification in 83% of the cases with known origin. GEP correctly classified 94% of ACUP in which IHC identified the primary site, and added valuable information in 64% of the cases that could not be classified by IHC [144]. A different study assessed the feasibility of a 10-gene reverse transcriptase polymerase chain reaction assay to identify the tissue of origin in patients with CUP. The assay was technically feasible in 87% of patients and a tissue of origin could be identified in 61% of patients. This assay was designed to detect six tumour types, and in cases where GEP detected one of those cancers the clinical and pathological features were mostly consistent with these diagnoses. As expected mainly patients with CRC benefited from a more detailed primary tumour characterization as treatment regimens used for CRC differ from platinum/taxane duplets often applied in CUP [145]. Thus, ACUP with a CRC profile is an example of a particular CUP subset that benefits from specific therapy [146].

Whether the considerable technical requirements for molecular assignment of tissue of origin in cancer of unknown primary will translate in improved treatment responses and prolonged survival however remains to be shown [147]. With the availability of molecular targeted treatment options this might change though and molecular diagnostics and biomarker identification could concomitantly serve to (*i*) establish a tissue of origin profile and (*ii*) determine a treatment modality of first choice including targeted agents.

Prognostic relevance of gene expression analysis: diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma in adults [148]. This disease is recognized as a distinct entity in the WHO classification of lymphoid neoplasms, but it has been acknowledged that DLBCL comprises genetically heterogenous lymphomas [149]. The introduction of the CD20-targeted monoclonal antibody rituximab into clinical practice has significantly improved progression-free and overall survival in all risk groups [148]. These risk groups previously have been defined by easily assessable clinical and laboratory prognostic factors (age, extranodal involvement, elevated lactate dehydrogenase, poor performance status, stage III/IV disease) [150]. More recent analysis by means of GEP identified two biologically and clinically distinct molecular subtypes of DLBCL. The germinal centre (GC)-like DLBCL subtype, that most likely arises from normal GC cells, and the activated B-cell-like DLBCL subtype that comes up from a post-GC B-cell blocked in further differentiation [151, 152]. With standard chemotherapy (CHOP: doxorubicin, vincristine, cyclophosphamide and prednisone) these two GEP-defined populations exhibit a significantly different clinical outcome [153]. A different GEP approach that also included DLBCL cases treated with CHOP discriminated four subgroups displaying prognostically distinct gene expression signatures: a 'GC' subgroup (favourable prognosis), a 'proliferation' subgroup (poor prognosis), a 'major histocompatibility class II' subgroup (inferior prognosis) and a 'lymph node' signature subgroup (favourable prognosis) [151, 154]. A third study identified genes implicated in DLBCL outcome that include B-cell receptor signalling, critical serine/threonine phosphorylation pathways and apoptosis [155]. Taken together, results from GEP indicate that in DLBCL pre-treatment gene expression signatures determine outcome. Several of the hereby identified biomarkers could be incorporated into routinely performed IHC or FISH analysis of lymphoma specimens. Examples for phase II/III clinical trials investigating drugs targeting molecular drug targets that were identified by GEP include enzastaurin, a protein kinase C-β inhibitor [155–157] and fostamatinib disodium, a Syk TKI that might specifically target DLBCL that express a 'B-cell receptor signalling' signature [157, 158].

The challenges in identification of novel prognostic biomarkers in DLBCL include robustness and reproducibility of the techniques (IHC and GEP), as well as availability at reasonable cost. The predictive value of new markers with respect to response to the currently available best treatment, in this case immunochemotherapy, needs to be established. The presumption that DLBCL is a curable disease should not prevent the inclusion of patients into clinical trials that assess new molecular targeted treatments as these treatments might well be associated with less side effects. GEP and other diagnostic tools should allow identification of patients suitable for a specific treatment.

The role of biomarker analysis within clinical trials – involvement of pathologists

Increasing knowledge of the biology of human cancer has provided an enormous number of interesting therapeutic target structures. Drug design has been facilitated by progress in structural analysis of putative target proteins [159]. Switching from chemotherapeutic agents to molecular targeted drugs however

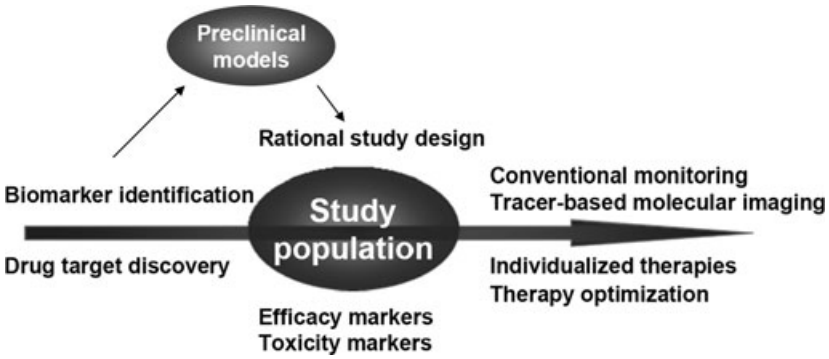

Fig. 3 Biomarkers analysis in molecular targeted therapy trials. The pathologist's role comprises drug target discovery and biomarker identification, as well as evaluation of toxicity and efficacy in normal and tumour tissue samples.

may require a different selection of study patients and a different assessment of optimal drug administration as well as efficacy (reviewed in [160, 161]) and this process involves pathologists at various stages.

Identification and validation of predictive biomarkers in trials evaluating molecular targeted treatments

Molecular targeted agents may fail in clinical trials due to an inappropriate study design that does not enrich a patient population that most likely will benefit from treatment due to the genetic and biological heterogeneity of specific tumour subgroups. Several approaches have been used to address this problem. First, in retrospective approaches, subgroups responding to a molecular targeted therapy in clinical trials have been further analysed for genetic or molecular characteristics associated with response, including predictive biomarkers established in other diseases entities. In the case of NSCLC and response to gefitinib, this evidence was derived from the finding of *EGFR* activating mutations identified in brain tumours. In NSCLC, sequencing the coding region of *EGFR* in gefitinib responders and non-responders revealed activating *EGFR* mutations in gefitinib responders [96]. This finding was taken as a basis for phase III trials examining first-line TKI treatment in patients with NSCLC harbouring activating EGFR mutations [98]. Second, in a less biased approach, it has been shown that genome-wide screening for receptor tyrosine kinase mutations is feasible [162]. In addition to histopathological and molecular assessment the strategy of selecting patients based on clinical characteristics has been proven efficacious. In the case of NSCLC Asian parentage, female gender, non- or ex-smoking status and adenocarcinoma histology were identified as specific epidmiological markers associated with response to EGFR TKI [95].

Interesting experimental data that may be integrated into prospective clinical trials in the future were derived from the screening of tumour cell lines using an 'integrated genomic profiling' approach [163]. Based on the presumption that distinct genetic lesions create a state of addiction to activated oncogenic signalling pathways [11], genomically annotated cell lines that represent primary tumours were used to screen compound libraries. *KRAS*-driven murine lung tumours were found highly susceptible to Heat shock protein (Hsp) 90 inhibitors and tumours with increased v-*Abl* and *SRC* copy numbers are greatly sensitive to the Src/Abl TKI dasatinib [163]. Thus, primary cancer genomics using acknowledged cell line profiles could be used to enrich suitable patient subgroups in clinical trials examining molecular targeted drugs in the future (Fig. 3).

Assessment of optimal drug dose, schedule and treatment combinations

Conventional cytotoxic drugs usually result in a therapeutic effect that is correlated with its toxic effect. The maximum tolerated dose (MTD) is therefore established in phase I clinical trials. This might not be a suitable approach when using targeted drugs since toxic effects and target inhibition could occur at different drug levels or through specific drug schedules [160, 164, 165]. A promising approach of a study design that tries to identify rationally based doses and schedules for targeted cancer treatment has been realized in pharmacodynamic phase I studies. Recent published work uses repeated tumour and normal tissue biopsies to assess the optimal dosage and scheduling of mammalian target of rapamycin (mTOR) inhibiting drugs [166, 167]. mTOR is aberrantly activated in various human cancers and controls cellular proliferation and metabolism [168]. By assessing the inhibition of mTOR downstream events (*e.g.* phosphorylation of S6 Kinase 1) in tumour biopsies during different dosing and scheduling of everolimus, the dosage-inhibiting mTOR activity was below the previously determined maximum tolerated dose and could be used in future trials [166]. Furthermore, by investigating additional pathways that might be activated upon inhibition of the targeted signal, rational targeted drug combinations can be identified [167]. Pharmacodynamic studies such as biomarker modulation using tumour tissue obtained before and after application of the study drug should therefore be included into early clinical trials whenever technically feasible and appropriate from an ethical point of view (Fig. 3).

Summary

Increasing knowledge about the molecular pathophysiology of tumour growth and metastasis and new techniques to screen and monitor molecular aberrations in patient's tumour tissues have greatly changed diagnosis, treatment and monitoring of tumour patients in the last decade. Neoplasias which have been previously diagnosed as single entities are now recognized as very heterogeneous and distinct malignancies with different molecular aberrations, pathophysiology and outcome. In haematological malignancies this has already led to tailored treatment strategies, which are based on molecular profiling. These personalized treatment strategies are now also applied to solid tumour patients, where oncologists to a very high extent rely on the analytical techniques provided by pathologists. For the treating oncologist and the pathologist knowledge of the molecular mechanisms of tumorigenesis in a particular patients is already today of upmost importance for accurate diagnosis and treatment in many cases. It is very likely that molecular profiling will further change cancer diagnosis and treatment dramatically in the near future. To keep pace with this development oncologists and pathologists have to cooperate very closely in diagnosis, treatment, monitoring and research. Pathologists should be already integral part in the design and planning of clinical trials with molecular defined modern cancer therapies.

Acknowledgement

We thank the staff of the III. Medical Department, Klinikum rechts der Isar, TU München, for ongoing critical discussion of clinical aspects of targeted therapy.

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