

## REVIEW

# A 2015 update on predictive molecular pathology and its role in targeted cancer therapy: a review focussing on clinical relevance

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In April 2013 our group published a review on predictive molecular pathology in this journal. Although only 2 years have passed many new facts and stimulating developments have happened in diagnostic molecular pathology rendering it worthwhile to present an up-date on this topic. A major technical improvement is certainly given by the introduction of next-generation sequencing (NGS; amplicon, whole exome, whole genome) and its application to formalin-fixed paraffin-embedded (FFPE) tissue in routine diagnostics. Based on this 'revolution' the analyses of numerous genetic alterations in parallel has become a routine approach opening the chance to characterize patients' malignant tumors much more deeply without increasing turn-around time and costs. In the near future this will open new strategies to apply 'off-label' targeted therapies, e.g. for rare tumors, otherwise resistant tumors etc. The clinically relevant genetic aberrations described in this review include mutation analyses of RAS (KRAS and NRAS), BRAF and PI3K in colorectal cancer, KIT or PDGFR alpha as well as BRAF, NRAS and KIT in malignant melanoma. Moreover, we present several recent advances in the molecular characterization of malignant lymphoma. Beside the well-known mutations in NSCLC (EGFR, ALK) a number of chromosomal aberrations (KRAS, ROS1, MET) have become relevant. Only very recently has the clinical need for analysis of BRCA1/2 come up and proven as a true challenge for routine diagnostics because of the genes' special structure and hot-spot-free mutational distribution. The genetic alterations are discussed in connection with their increasingly important role in companion diagnostics to apply targeted drugs as efficient as possible. As another aspect of the increasing number of druggable mutations, we discuss the challenges personalized therapies pose for the design of clinical studies to prove optimal efficacy particularly with respect to combination therapies of multiple targeted drugs and conventional chemotherapy. Such combinations would lead to an extremely high complexity that would hardly be manageable by applying conventional study designs for approval, e.g. by the FDA or EMA. Up-coming challenges such as the application of methylation assays and proteomic analyses on FFPE tissue will also be discussed briefly to open the door towards the ultimate goal of reading a patients' tissue as 'deeply' as possible. Although it is yet to be shown, which levels of biological information are most informative for predictive pathology, an integrated molecular characterization of tumors will likely offer the most comprehensive view for individualized therapy approaches. To optimize cancer treatment we need to understand tumor biology in much more detail on morphological, genetic, proteomic as well as epigenetic grounds. Finally, the complex challenges on the level of drug design, molecular diagnostics, and clinical trials make necessary a close collaboration among academic institutions, regulatory authorities and pharmaceutical companies.

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## INTRODUCTION

Targeted drugs, in particular therapeutic antibodies, kinase inhibitors and PARP inhibitors, have started to transform current treatment strategies in oncology and new organizational structures, which we have started to discuss previously and which we now review in more detail.<sup>1</sup> Prior to application almost all of them require a so called pre-therapeutic companion diagnostic test to identify certain molecular alterations. More precisely, a well-defined biomarker, most often a characteristic genetic alteration or particular protein (over-)expression, that indicates the efficacy of the respective drug has to be identified in the tumor tissue of individual patients. The tissue-based analysis is mostly done by

predictive molecular pathology applying conventional or high-throughput techniques on FFPE tissue. This is the basis of personalized, individualized or precision medicine.

This new and extremely exciting field of medicine creates a number of challenges which can be managed only by new ways of thinking and new organizational structures. Some examples are

1. The scope of the interdisciplinary tumor board that consists of radiologists, pathologists, oncologists, surgeons and organ specialists, needs to be extended to account for the increasingly available clinically relevant molecular profiling information by including specially trained molecular pathologists.

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- It is pivotal to further integrate 'molecular knowledge' into diagnostic pathology training.
- Due to the complexity of new combination treatment new designs of clinical studies have to be defined and approved by the regulatory authorities.
- The classification of malignant tumors may change considerably by introducing a molecular classification of malignant tumors. One example is the molecular subtyping of breast carcinomas. The up-coming edition of the WHO Blue Series on brain tumors will include new entities, which are defined mainly or only by molecular characteristics.

Despite the increasing importance of molecular information for tumor classifications, examples such as the different druggability of BRAF V600E mutations in melanoma compared to colorectal cancer indicate that for the foreseeable future the classical morphology-based WHO classification will remain in place but, will certainly have to be supplemented by genetic, proteomic metabolomic and other data so that finally the combination of all the techniques and the provided data will lead to an optimal strategy for patient care.

Clinical trial design—the combinatorial complexity challenge in precision oncology and predictive pathology

Actionable mutations indicating a response to targeted therapy are often only present in a minority of tumors. EML4-ALK translocations, for instance, are found in only about 4% of all NSCLC, which poses a major challenge for patient recruitment in clinical trials. The clinical study that demonstrated the efficacy of Crizotinib in EML4-ALK-positive NSCLC comprised 105 sites in 21 countries only to recruit 347 patients.<sup>2</sup> This comes as no surprise as the low frequency of EML4-ALK required about 10,000 NSCLC patients to be screened. To address this challenge, alternative trial designs have been proposed, such as the so called 'basket' or 'umbrella' trials, which recruit patients with different cancers but identical mutations or vice versa.<sup>2,3</sup> While this partially solves the low frequency problem in a monotherapy setting, the situation becomes more difficult when it comes to combination therapies, which will likely become necessary because of frequent resistance development against single drug therapies. Although only a relatively small proportion out of the hundreds of genetic aberrations found in most cancers (over 800 in an average squamous cell lung cancer<sup>4</sup> are currently therapeutically targetable, the list of actionable mutations and corresponding drugs is constantly growing). Imagine a rather conservative scenario with only 10 mutations: already here, 45 alternative 2-drug combination therapies exist. In only moderately more complex situations,

such as selecting a triple-therapy against 3 out of 20 mutations, over 1,000 alternatives exist, a number that increases to nearly 10,000 for 40 possible targets. Although the number of possible combination therapies may be reduced to some extent based on knowledge on pathway biology and clinical experience, multiple drug combinations that would have to be compared in clinical trials remain. This complexity is practically not addressable with existing clinical trial strategies especially when seen in conjunction with the low frequency challenge described above. As an example, when a triple-therapy against 3 actionable mutations that each occur in 20% of the tumors is to be evaluated in a clinical trial, 25,000 patients would have to be screened to recruit just 200 patients.

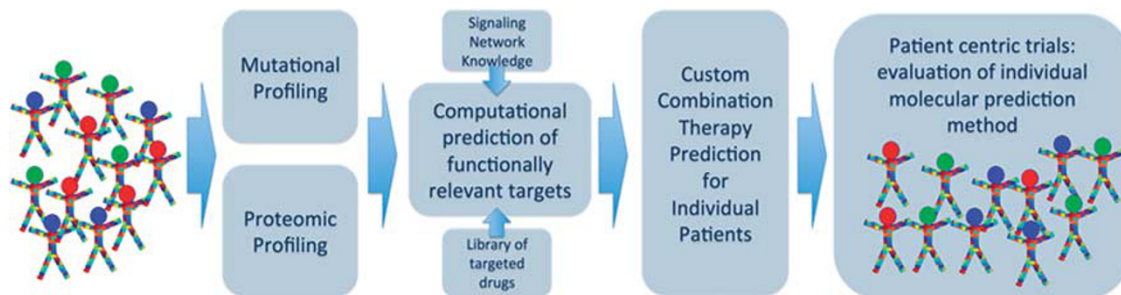
A solution to these challenges requires novel, more patient-centric clinical trial design strategies, similar to approaches implemented in the so called N-of-1 trials.<sup>5</sup> Such trials would among other things rely on a—compared to static genomic profiling—more functionally oriented systems medicine approach based on proteogenomic analyses and bioinformatic modeling (Figure 1). Predicting the functional relevance and efficacy of a therapy for individual patients would help avoid systematic or random testing of alternatives. Novel patient-centric clinical trials would then not just evaluate a particular therapy for a given mutational signature, but assess the capability of the predictive molecular pathology approach to design a custom-tailored therapy for an individual patient<sup>6,7</sup> for a more detailed discussion.

## METHODOLOGICAL CONSIDERATIONS

Since formalin fixation and paraffin embedding (FFPE) is and will be the by far the most widely used method for tissue fixation in the routine diagnostic setting the high-throughput technologies have been adapted to the particular quality of DNA and RNA in FFPE tissue. This opens the door to extended molecular analyses of almost all tissues. It has to be emphasized that carefully controlled pre-analytic steps, standard operating procedures (SOPs) and bio-mathematic knowledge are of absolute importance. In addition the labs working in clinical diagnostics should constantly perform external quality controls.

Next-generation sequencing and liquid biopsy

Next-generation sequencing (NGS) has recently been adapted to diagnostic requirements and a growing number of diagnostic laboratories and companies offer a variety of NGS-based services. However, different NGS-based approaches ranging from amplicon-based (targeted) NGS, whole exome (WGS) or whole genome NGS (WES) exist and are, as yet, suited for different applications. With respect to tumor diagnostics in molecular



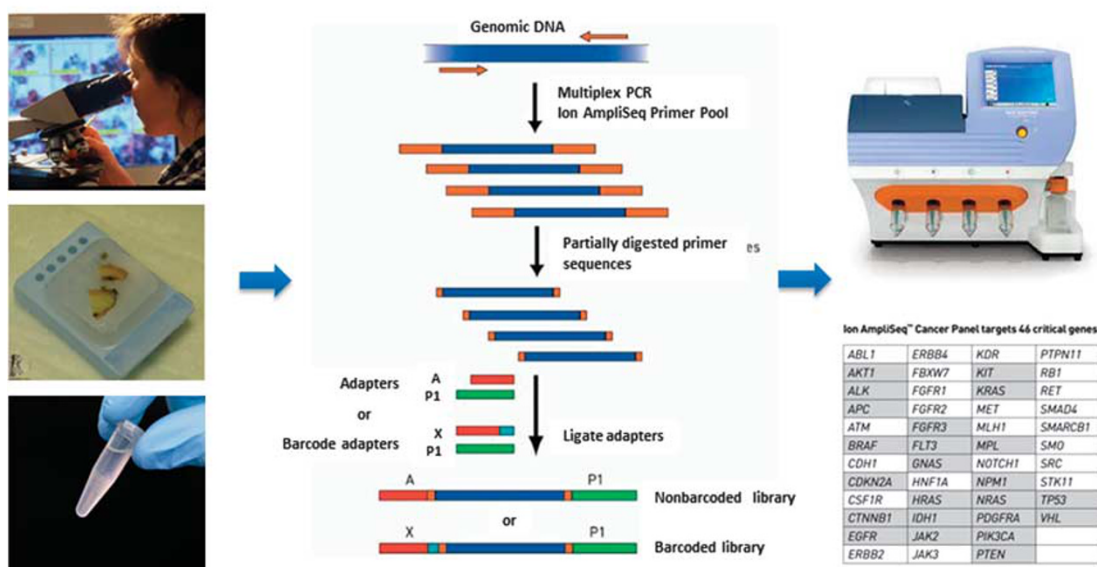
**Figure 1.** Proposal for a systems medicine approach to patient-centric precision oncology trials. Conventional clinical trial design is incompatible with the high combinatorial complexity of targeted combination therapy selection even for moderate numbers of actionable mutations found with next-generation sequencing. However, additional proteomic profiling enhanced by computational modeling facilitates the functional evaluation of molecular aberrations and may be used to predict personalized therapies for individual patients. The proposed trial design no longer tests a particular drug for a defined cancer in a homogeneous cohort of patients, but evaluates the method used to predict the custom-tailored therapies for individual patients.

pathology predominantly amplicon-based NGS is applied. This is due to the fact that (i) the vast majority of sequence information generated by WGS and WES cannot be translated into clinical intervention, (ii) WGS and WES are not ready for FFPE based NGS, (iii) bioinformatics for WGS and WES data requires both, germline and tumor data, (iv) the coverage of WGS and WES appears to be too low especially in cases of low tumor cell content, (v) the turn-around times of WGS and WES are too long in a diagnostic setting and (vi) the costs (consumables, data analysis, bioinformatics and data handling) of WGS and WES are much too high. In contrast, targeted amplicon-based NGS strategies can overcome most of these disadvantages. Since the first reports that small amounts of FFPE-derived DNA are feasible for NGS-based analyses<sup>8</sup> several protocols have been established that both adapt amplicon length as well as DNA extraction methods to the specific requirements of FFPE tissue and biopsy samples. The restriction of the gene panels employed for amplicon-based to targets with clinically actionable mutations enables relatively small amplicon numbers. In turn, the small number of amplicons provides very high coverages despite intensive multiplexing (parallel processing of a considerable number of samples), which thus is very cost effective. The resulting high coverage provides enough sensitivity even in situations of low tumor cell content or tumor cell heterogeneity and provides the basis for thresholds to ignore artifacts introduced by formalin fixation. Finally, due to the restriction to known druggable genes, the bioinformatics analysis of amplicon-based NGS data is feasible with commercial software products and basic bioinformatic knowledge. Regarding the turn-around-time, amplicon-based NGS can be very much adapted to the needs and the sample numbers of the respective molecular diagnostic laboratory (Figure 2). This provides processing times per sample which are very similar to those accepted for conventional Sanger- or pyro-sequencing.

A question which was extensively discussed concerns the reliability of amplicon-based NGS for diagnostic purposes since Sanger sequencing or pyro-sequencing was regarded as gold standard since many years. Amplicon-based NGS—despite less

complex as compared to WGS and WES—is a comprehensive approach because of the simultaneous generation of many amplicons which are subsequently individually sequenced in parallel by NGS. As a result, each amplicon is represented by a huge number (several hundreds up to thousands) of individual sequences which may differ at various nucleotide positions. Furthermore different methods exist to generate the multiplex amplicons (pull-down enrichment, primer extension and ligation or multiplex PCR) as well as to produce the sequences (mainly measurement of fluorescent dye incorporation and imaging (Illumina) or semiconductor sequencing (ThermoFisher). In addition to the NGS methods, there are further steps in the entire procedure, which might interfere with the sequencing results such as DNA extraction, the primer composition of the gene panels and the bioinformatics interpretation. To clarify the comparability of the data NGS data produced at different sites and applying various methods and techniques we initiated a multi-center study in the context of the German Consortium for Translational Cancer Research (DKTK). Based on molecular predefined tissue samples (Sanger sequencing) and cell line dilutions we produced Illumina MiSeq data and Life Technologies PGM data at 5 different pathology centers using exactly the same samples. Although the number of sequence reads per amplicon obtained for the regions of interest differed due to the local design of the sequencing approach, the identification of the predefined mutations was successfully achieved at all sites involved and by all NGS methods applied independent from the gene panels used (manuscript in preparation). However we recognized a significant impact of the DNA extraction method used which may cause significant differences in the allelic frequencies of the detected mutations or even complete drop-out of some cases especially in combination with the respective NGS technique used. In conclusion, it should be kept in mind that amplicon-based NGS is a complex approach which has to be carried out, controlled and supervised by trained technicians, scientists and pathologists in order to avoid the production of insufficient data and a misinterpretation of the results. Therefore the setup of quality assurance schemes

## Integrating Next Generation Sequencing in Diagnostic Pathology



**Figure 2.** Integration of next-generation sequencing in diagnostic pathology exemplified by the Ion AmpliSeq technology. This and others procedure proved to be very valuable in molecular analyses of FFPE tumor tissue. This is of particular relevance in predictive molecular pathology as prerequisite of targeted therapy of cancer.



taking these new technologies into account are required as soon as possible. Under these preconditions amplicon-based NGS is ready to step into the routine in molecular pathology. In line with this in late 2013, with the Illumina MiSeqDx the first next-generation high-throughput sequencer was approved by the FDA (Food and Drug Administration) thus paving the way for the application of numerous genome-based tests.<sup>9</sup>

Apart from its value in molecular pathology, the high sensitivity of amplicon-based NGS now enables non-invasive analyses such as early detection of relapse-determining mutations in blood or plasma samples from patients under treatment.<sup>10</sup> This process, today commonly addressed as liquid biopsy, has led to an improved understanding of the development of therapy resistance and opened the path for a future monitoring of cancer patients.<sup>11</sup>

#### Perspectives on proteomics: the next level of molecular tumor profiling?

Pan-cancer mutational profiling efforts by the consortia TCGA (the cancer genome atlas) or ICGC (international cancer genome consortium) and many other groups have demonstrated a high complexity of the mutational landscapes in most cancers. The average squamous cell lung cancer, for instance, has been shown to harbor over 800 genetic aberration including exon mutations, copy number variations and rearrangements.<sup>6</sup> Although only a minority of the observed mutations is believed to belong to the group of driver mutations that are causally linked with cancer pathology, the functional and therefore clinical implications of many less frequent mutations for individual patients are largely unknown. And even if a (rare) mutation is known to affect protein function, in principle, modulations of such effects through other mutations or epigenetic as well as posttranscriptional and posttranslational regulation may differ substantially among tumors. A solution would be to complement genomic with proteomic profiling of tumor tissue to help relate mutational profiles to protein changes as more direct indicators of tumor cell function. Proteomic tests have already been shown to provide prognostic information, on—as, for example, in the case of Veristat (Biodesix)—the efficacy of EGFR inhibitory therapy on NSCLC.<sup>12,13</sup> However, while a step towards a better characterization of tumors, this mass spectrometry based test of patient serum relies only on eight abstract features from the obtained mass spectra that were correlated statistically with clinical outcome. To achieve a better understanding of the mechanisms underlying cancer pathology and to facilitate therapy decision making based on causality rather than statistical correlations more comprehensive and functional characterizations are needed, including protein expression and, as one of the major regulatory mechanisms of signaling pathways, protein phosphorylation measurements. Antibody-based protein array techniques or pathway profiling kits are already commercially available, shotgun or targeted mass spectrometry based proteomics are technically more complex. However, the advantage of mass-spec based approaches lies in providing a more global (simultaneous measurements of several thousand proteins) and unbiased view of the functional implications of mutational profiles and will likely play an important role in future molecular tumor profiling.

#### External quality assessment (EQA)/quality control (QC)

EQA is a systematic process for the assessment of diagnostic and predictive tests. A number of test samples are distributed to the participating centers for analysis. Within these trials participants may or may not certify for different diagnostic applications (eg estrogen/progesteron receptor, HER2, Ki67, EGFR, RAS, ALK). The goal is to achieve a high level of accuracy, standardization and reproducibility among different centers.<sup>14</sup> The QuIP initiative ('Qualitätssicherungs-Initiative Pathologie') serves as an example

of QC at the national and European level. Here, ALK-QCs might serve as an example: The first (2012) showed as success rate of 60.3% (32 of the 53 participating institutes were certified),<sup>15</sup> in the second (2014) 92.5% (37/40) succeeded, demonstrating a successful learning curve.

A new challenging QC initiative started by a consortium of Institutes of Pathology in Germany is dealing with amplicon-based next-generation sequencing (NGS) for detection of pathogenic mutations in the genes for BRCA1/2. This became relevant since a new PARP inhibitor was approved for the treatment of high-grade serous ovarian and peritoneal carcinomas if a pathogenic BRCA1/2 mutation could be demonstrated in the tumor tissue of the patients. The method of choice for this purpose in amplicon-based NGS although other sequencing techniques are not excluded.

To open EQA to a broad community of pathologists in June 2014, a joint agreement was signed by the German Society of Pathology, the Association of German Pathologists and the European Society of Pathology. Each organization accepts the other's quality assessment programs.

#### MOLECULAR DIAGNOSTICS OF TUMORS (MOLECULAR CLASSIFICATION)

Clinicians' demand in tissue-based diagnostic analyses of solid and hematological tumors is becoming more and more challenging. This is in particular true if therapeutical options need companion diagnostics. While pathology reports on tumor type, WHO-code, pTNM status, dignity, i.e. benign, malignant, *in situ* carcinoma or borderline lesion; histogenesis and prognostic remain the skeleton of tumor classification the predictive relevance is limited. This means conventional histopathology has to be refined by molecular data often very relevant for selecting the optimal treatment. The most relevant examples are given below.

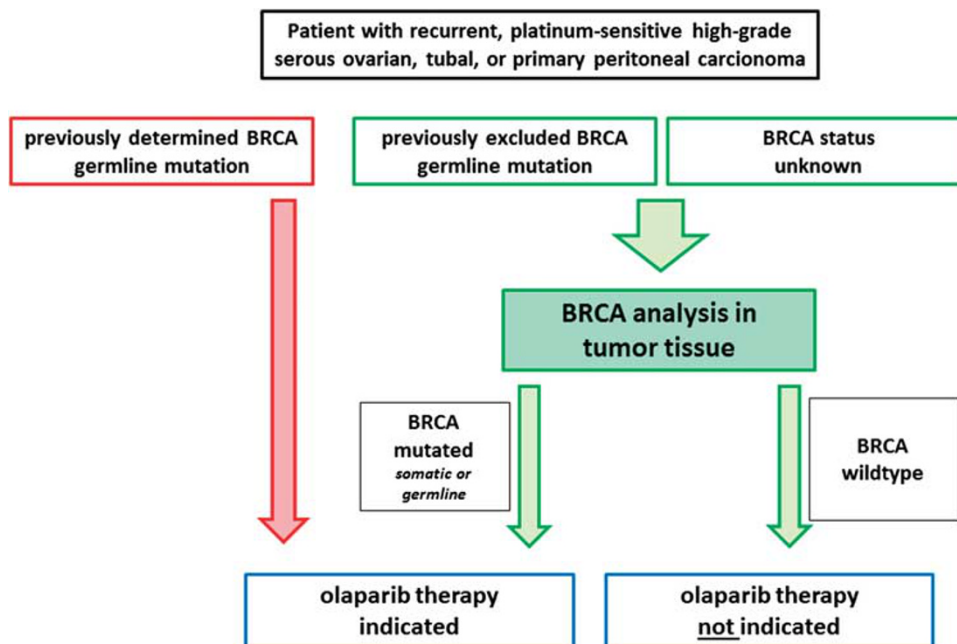
##### Breast cancer

In breast cancer, the classical morphology-based classification has been largely replaced by the molecular classification.<sup>16</sup> Based on gene expression profiling as well as immunohistochemistry, at least five different subtypes can be distinguished: luminal-A, luminal-B/HER2 negative, luminal-B/HER2-positive, HER2-positive/non-luminal and triple negative. At the St Gallen conference 2011 the molecular subtypes were introduced as the many classification system for clinical decisions in breast cancer, this classification was slightly modified at the St Gallen 2013 meeting.<sup>17</sup>

Breast cancer subtypes can be determined by gene expression analysis. However, in clinical practice, the standard approach is the immunohistochemical investigation of estrogen receptor, progesterone receptor and HER2. The differential diagnosis of luminal-A versus luminal-B tumors can be a challenge in some situations. It has been suggested to use Ki67 as a marker for luminal-A versus B tumors, however, the cut-points for Ki67 are still under debate and international efforts for further standardization are still ongoing.

In the last years, several diagnostic assays have been developed to define a low risk group of luminal-A tumors that have an excellent prognosis with endocrine therapy alone. The OncotypeDX<sup>18</sup> assay, the Endopredict<sup>19</sup> assay as well as the PAM50/Prosigna<sup>20</sup> test are based on mRNA analysis of gene expression in formalin-fixed paraffin-embedded tissue and can be used to defined low risk tumors that might not need a chemotherapy treatment. In addition, some of the tests (Endopredict<sup>21</sup> and Prosigna<sup>22</sup>) have been used to identify which patients would benefit from extended endocrine therapy of more than 5 years.

In addition to mRNA-based gene expression profiling, mutation-based classification are under evaluation in breast cancer. The most commonly mutated genes are p53 and PIK3CA. For PIK3CA, it has recently described that tumors with PIK3CA mutations have



**Figure 3.** Algorithm for predictive BRCA testing in tumor tissue. Patients with recurrent, high-grade serous ovarian, tubal, or primary peritoneal carcinoma may be considered for an olaparib maintenance therapy. For patients with unknown BRCA status or patients who have previously been tested negative for a BRCA germline mutation BRCA status should be determined in tumor tissue, which enables the detection of germline and somatic mutations (green). Patients in whom a tumoral BRCA mutation is detected are eligible for therapy. Patients who have previously been tested positive for a germline BRCA mutation are eligible for therapy and do not need further testing (red).

a reduced response to neoadjuvant anti-HER2 therapy, further validation studies are ongoing.<sup>23</sup>

#### Ovarian cancer

Sequence analysis of the breast and ovarian cancer susceptibility genes BRCA1 and BRCA2 genomic status is the first routinely performed molecular test for ovarian cancer. According to data provided during The Cancer Genome Atlas (TCGA) project high-grade serous carcinoma, which is the most frequent histological subtype of ovarian cancer (70%), reveals germline mutations within BRCA1 or BRCA2 in approximately 20% of primary tumors, additional 6% show somatic mutations.<sup>24</sup> BRCA mutations lead to a homologous recombination deficiency (HRD), which is characterized by a reduced ability to repair DNA double strand breaks.<sup>25</sup> The inability to perform DNA repair adequately is the cause of an increased cancer risk on the hand, but also the reason for the high sensitivity of BRCA mutated tumors to agents that induce DNA single strand breaks and thereby produce a synthetic lethality. Particularly, BRCA mutated ovarian cancer is highly sensitive to platinum-based chemotherapy, which seems to be the reason for the comparably favorable prognosis of this molecular tumor subtype.<sup>26</sup> Poly(ADP-ribose) polymerase inhibitors (PARPi) are the most recently approved targeted therapeutics for ovarian cancer. In January 2015 the European Medicines Agency (EMA) has approved the PARP inhibitor olaparib (Astra Zeneca) for maintenance therapy of recurrent platinum-sensitive high-grade serous ovarian, tubal or peritoneal carcinoma revealing germline or somatic BRCA mutations. The approval was based on a phase II randomized clinical trial, which showed a significant progression-free survival (PFS) advantage in the olaparib vs the placebo arm (median PFS 8.4 months vs 4.8 months,  $P < 0.001$ ).<sup>27</sup> The survival advantage was most pronounced in BRCA mutated carcinomas (median PFS 11.2 months vs 4.3 months  $P < 0.0001$ ), however, response was also seen in patients with BRCA wild-type status

(median PFS 7.4 months vs 5.5 months,  $P = 0.0075$ ).<sup>28</sup> This points to the possibility that apart from BRCA genomic status other molecular aberrations—most likely those causing HRD (also referred to as ‘BRCaness’)<sup>29</sup> might be involved in the response to PARPi, however, BRCA mutations being the most frequent and best studied mechanism underlying HRD, BRCA sequence analysis is the companion diagnostic test for olaparib to date. Because of the occurrence of somatic mutations, analysis of tumor tissue in patients who have not yet been tested positive for a hereditary BRCA mutation (both patients with wild-type and unknown germline status) is the most sensitive method to detect all patients suitable for an olaparib maintenance therapy (Figure 3). The reported rates of somatic mutations vary, depending on the sample size and selection criteria of the cohort investigated, however is likely to be higher in patients with platinum-sensitive high-grade serous cancer than in the total population of ovarian cancer patients (10–14%).<sup>30</sup> Both genes are of considerable size (BRCA1: 24 exons, 5,592 bases; BRCA2: 27 exons, 10,257 bases) and although some mutational hotspots occur, most mutations are widely distributed along the exome rendering the sequence analysis challenging (<http://cancer.sanger.ac.uk/cosmic/gene/overview?ln=BRCA1>; <http://cancer.sanger.ac.uk/cosmic/gene/overview?ln=BRCA2>). Next-generation sequencing (NGS) techniques, which are increasingly used in molecular pathology, are therefore the methodological option for BRCA analysis in a routine diagnostic setting. Apart from the need to sufficiently cover the BRCA1/2 exome during sequence analysis, the interpretation of detected variants requires a high bioinformatics expertise, as multiple polymorphisms exist in each given individual, necessitating a functional and clinical annotation of each variant. Several publicly available databases are in use for clinical annotation (eg the Breast Cancer Information Core (BIC) of the NIH), and are until now based on the estimation of familial breast and ovarian cancer risk. Due to the very recent approval of PARPi for ovarian cancer treatment, no robust data according to the predictive capacity of

particular sequence variants for response to PARPi exist to date, however, those data are expected to accumulate with the clinical use of PARPi within the next years.

### Colorectal cancer

Antibody mediated blockade of the epidermal growth factor receptor (EGFR) is a therapeutic option in the treatment of advanced colorectal cancer. In several clinical trials it was shown, however, those only patients with cancers bearing no mutation in the KRAS gene benefit from EGFR-targeting antibodies like cetuximab and panitumumab.<sup>31,32</sup> KRAS is an intracellular downstream component of the EGFR signaling cascade. Mutations of the gene result in a constitutional activation of the EGFR pathway and thus might completely abolish the effects of an upstream inhibition of EGFR.

In 2008, therefore, exclusion of KRAS hot spot mutations in exon 2 have become mandatory for the application of EGFR-targeting antibodies in the first-line therapy of advanced colon cancer. The list of genetic alterations to be excluded for EGFR antibody therapy has been extended to KRAS exons 3,4 and NRAS exons 2–4 in 2013 after non-responsiveness has also been found for these alterations.

Other genetic changes like BRAF and PI3K mutations have also been described to associate with lacking EGFR antibody response<sup>33</sup> but the data generated in a different study<sup>34</sup> is too controversial to include these markers in routine predictive molecular testing and therapy decision making to date. It is foreseeable, however, that the number of markers predicting therapy response in CRC will rise with novel data generated from larger CRC cohorts and with the introduction of novel therapy strategies which may include antibody targeting of BRAF in the near future.

Another application of molecular pathology in CRC refers to a principal genetic mechanism causing the disease. In approximately 10–15% of CRCs microsatellite instability (MSI-H), a consequence of DNA mismatch repair deficiency, is found. MSI-H is a hallmark alteration of HNPCC/Lynch syndrome-associated tumors, but is also found in sporadic colon cancers.<sup>35–37</sup> MSI-H has a significant impact on tumor biology. This is reflected by a more favorable prognosis of this molecular CRC subtype and a lacking response of MSI-H CRCs to 5-fluorouracil monotherapy.<sup>38</sup>

To date, knowledge of the MSI status is required to address two routine applications/requirements:

1. Following the revised Bethesda guidelines,<sup>39</sup> colon cancer is tested for MSI in order to evaluate the possibility of Lynch syndrome. In combination with immunohistochemical analysis of the mismatch repair proteins (MLH1, MSH2, PMS2, MSH6) and EPCAM<sup>40</sup> the molecularly determined MSI status guides further genetic counseling.

2. Moreover, the MSI status is determined in poorly differentiated cancers according to conventional histologic grading. Only in the absence of MSI-H, poor histologic/high-grade differentiation (G3–4) is considered a prognostic marker while MSI-H cancers are considered low grade, irrespective of the conventional histologic appearance.

### Non-small cell lung cancer (NSCLC)

To date the detection of EGFR- and ALK-alterations is the standard of molecular testing in NSCLC but should be further completed by testing for other markers as ROS1, MET or even KRAS under certain conditions.<sup>1,41–43</sup>

EGFR and ALK mainly occur in advanced-staged adenocarcinomas (ADC), whereas the frequency in squamous cell carcinomas (SCC) is very low.<sup>44</sup> However, under certain conditions (eg young patient, non-smoker) testing should be considered in the latter, as well.<sup>44</sup> KRAS- and EGFR-testing is performed by PCR (more and more NGS), ALK-, ROS1- and MET-testing mainly by Fluorescence

*in situ* hybridization (FISH).<sup>41,45,46</sup> Recent studies demonstrated, that a 'carefully validated' ALK-immunohistochemistry (IHC) is eligible for multi-center routine testing<sup>15,46,47</sup> at least as a screening tool and in samples that may be unsuitable for the FISH approach (eg very small amount of tumor cells, high amount of osseous tissue).<sup>48</sup>

KRAS-alterations (chromosome 12) are quite frequent (~30%). To date, "only" blockage of downstream kinases in the RAS-RAF-MEK-ERK pathway is under investigation, e.g. with the MEK1/MEK2-inhibitor selumetinib in combination with docetaxel.<sup>49,50</sup> However, to date a targeted KRAS-therapy is not available.<sup>49,50</sup> As mutual exclusiveness is discussed for KRAS, EGFR, ALK and ROS1, some authors suggest upfront KRAS testing, with further broad testing in a KRAS wild-type situation only.<sup>48–50</sup> In our opinion this testing approach costs tissue and time (as results should be reported within 5–10 days<sup>43</sup>). Furthermore, comutations (even if very rare) might occur and single patients could get lost for a promising therapy.

For patients with EGFR (~15%, chromosome 7) activating mutations (exon 19 deletion and exon 21 L858R point mutation) an EGFR-TKI (eg erlotinib, gefitinib or afatinib) is standard first-line therapy if a curative tumor resection is not possible.<sup>48–51</sup> Most EGFR exon 20 insertion mutants are resistant to EGFR inhibitors, except the A763\_Y764insFQEA, which is sensitive to erlotinib and gefitinib.<sup>51,52</sup> Secondary resistance to EGFR inhibitors may occur due to an additional EGFR-mutation (T790M) or MET-amplification,<sup>49,51,53</sup> therefore testing for the latter (see below) becomes more and more standard and combination of EGFR-TKI and MET-inhibitors are discussed.<sup>49–51</sup>

For patients with ALK-inversions/translocations (~3%, chromosome 2) the ALK-TKI crizotinib reached approval in 2011 (FDA) and 2012 (EMA).<sup>43–46</sup> Meanwhile "second generation" ALK inhibitors have been invented, facing the question of brain metastasis and ALK-resistance.<sup>48–50</sup> Acquired ALK-resistance occurs due to secondary ALK-mutations, ALK-amplification, as well as EGFR-, cKit- or KRAS-mutations.<sup>54,55</sup> Therefore, rebiopsy in these patients is highly recommended as this will have further impact on the consecutive therapy management.<sup>49,50</sup>

However, not only acquired secondary mutations but especially tumor heterogeneity is an highly important issue discussed in mechanisms of therapy resistance. Patients with a heterogeneous EGFR profile showed less response to gefitinib, as well as an earlier tumor progress and a shorter median survival.<sup>56</sup> These kind of aspects (eg major clones and subclones) should be further kept in mind when comparing results of biopsy and rebiopsy. Additionally, an evolving field is the so called 'liquid biopsy' encompassing the detection of circulating tumor cells (CTC), circulating tumor DNA (ctDNA), circulating RNA or microRNAs. To date, further robust validation and large independent prospectively designed comparative trials need to be done.<sup>57</sup>

Interestingly the above mentioned drug crizotinib (originally designed as MET-inhibitor) is an ALK/ROS1/MET-inhibitor that also showed good response data in patients with ROS1-translocation and MET-amplification.<sup>58,59</sup> Recently the FDA granted breakthrough therapy designation for crizotinib in ROS1-positive NSCLC. ROS1-testing (chromosome 6, translocation, ~0.5–2%, mainly ADC) is performed by means of FISH.<sup>41,49,60</sup> IHC and PCR are possible, however, (so far) not multi-center standardized.<sup>50,61</sup>

Testing for MET (chromosome 7, mutation/amplification/over-expression ~10%) is not only an option in the context of EGFR-resistance as described above,<sup>49,62</sup> as therapy response was shown with ALK-TKIs and MET-inhibitors in patients with MET-amplification and alterations at MET exon 14.<sup>59,63,64</sup> However, a clinical phase III trial (MET-inhibitor) was stopped recently and its role will need further evaluation.<sup>65</sup>

MET alterations are more frequent in ADC than in SCC, at the moment diagnosis is mainly made by FISH (different scoring systems!) and IHC (be aware of discordant IHC/FISH results!).<sup>60</sup>



To summarize, at the moment EGFR, ALK and ROS1 are in the focus of ADC-NSCLC testing and new drugs targeting even more than one alteration are under investigation (for an overview please see and read Table 1, page 441 in ref. 66).

Further alterations<sup>41,50</sup> that should be considered (encouraging studies) in case of negativity of the above described markers are:

- RET (chromosome 10; inversion/translocation; 1–2%, FISH).
- HER2 (chromosome 17; Exon 20 insertion, deletion, 1–2%, PCR, but not protein expression as known in breast and gastric cancer).
- BRAF (chromosome 7, activating point mutation V600E, fusion protein, 1–3%, PCR).

Thus parallel testing for EGFR, ALK and ROS1 encompassing an ALK-IHC seems a pragmatic manner. Figure 4 combines the markers discussed here in the context of a local (reflex) testing approach.

So far the data for SCC are not as encouraging as for ADC: The main two targets to test for are FGFR1 (chromosome 8, 20%, amplification, only 1% in ADC, detection by FISH) and DDR2 (chromosome 1, point mutation, 2%, PCR).<sup>50</sup>

The implementation of NGS in the daily routine might bring us different mutations in one tumor (not only concerning the above mentioned targets) in the context of tumor heterogeneity. This will not only broaden the spectrum of targeted therapy but will also help to better understand the questions of drug resistance.

#### Malignant melanoma

The incidence of malignant melanoma has increased rapidly in the past few years, partly due to changes in diagnostic criteria and improvement of screening methods, but also due to behavioral changes such as increased exposure to UVR.<sup>67</sup> A first breakthrough in the therapy of malignant melanoma was achieved by the approval of several targeted therapies for patients with BRAF mutated metastatic melanoma, Vemurafenib and later Dabrafenib.<sup>68,69</sup> About 50% of metastatic melanoma harbor a mutation in the oncogene BRAF, with V600E being the most common one (75% of V600 mutations), followed by V600K (20%) and V600R.<sup>70</sup>

The first results of the BRIM3 study revealed a prolonged overall survival of 13.9 months (vemurafenib) compared to 9.6 months (dacarbazine) in BRAF mut patients.<sup>68</sup> One of the biggest challenges in the therapy of BRAF mut patients with these targeted therapies is still the primary and secondary resistance which most patients develop over time. The success of the therapy is often restricted by the activation of the MAPK signaling pathway in BRAF WT cells and the resulting activation of proliferation followed by a fast relapse.<sup>71,72</sup>

Thus, the most important prerequisites for an optimal therapy of the patients, now and in future, are reliable and reproducible methods for BRAF testing and the development of new treatment strategies, particular by combining different approaches, to increase therapy success and to achieve long-term responses.

**BRAF testing.** Today, a multitude of different methods for BRAF testing is available, among them different sequencing technologies and real time PCR assays. The so called “next-generation sequencing” (NGS) became popular in the past few years, allowing for testing of multiple markers in one run, and will also become of interest for malignant melanoma due to the increasing requirements for molecular testing.

Furthermore, BRAF testing can also be done by immunohistochemistry.<sup>73</sup> However, the currently available antibody only allows for the testing of BRAF V600E mutations and thus should only be used as a screening method, combined with a second

technology for a subsequent analysis of all negative cases for mutations other than V600E.

**NRAS and KIT.** Besides BRAF mutations malignant melanomas harbor several other oncogenic alterations, like mutations in the GTPase NRAS and the tyrosine kinase KIT. In about 20% of all malignant melanoma an activating mutation in NRAS can be found which is mainly associated with a worse prognosis.<sup>74</sup> Since NRAS cannot be targeted directly, testing for NRAS alterations in malignant melanoma initially became of interest as a potential predictive factor for downstream applications e.g. for MEK inhibition.

KIT mutations in malignant melanoma are rare. About 15% of malignant melanoma harbor KIT mutations,<sup>75</sup> mainly in exon 11 and exon 13. KIT inhibitors like Sunitinib, Dasatinib and Imatinib, although primarily not developed for the treatment of malignant melanoma, turned out to be interesting therapy options for patients with KIT positive melanoma.

**Combinatorial therapies.** Due to the limited long-term effect of BRAF inhibitors, efforts are being made to combine different strategies to improve therapy outcome. One promising approach is the complete inhibition of the MAPK pathway by combining BRAF and MEK inhibitors. The first results of a Phase I/II study for the combination of dabrafenib and trametinib showed an increase of median PFS from 5.8 with dabrafenib alone to 9.4 months with the dabrafenib plus trametinib and showed even less toxicity compared to the treatment with one of the agents alone.<sup>76</sup> Furthermore, the use of ERK inhibitors is being tested for patients with MAPK pathway dependent resistance to RAF or MEK inhibitors.<sup>77</sup>

Recently, the newly-discovered approach of treatment with targeted therapies combined with novel immunotherapies, e.g. with the anti-CTLA4 antibody ipilimumab or anti-PD-1 and –PD-L1 antibodies nivolumab and lambrolizumab showed great promise for a prolonged therapeutic efficacy.<sup>78</sup>

**Cancer of unknown primary (CUP).** In about 5% of tumors the anatomical site of origin cannot be determined despite extensive examinations.<sup>79,80</sup> These cancers are termed “cancer of unknown primary”, CUP. Whether CUP represents a group of cancers with common biologic features, irrespective of the site of the primary tumor or if each CUP retains the signature of the tissue it developed from and which can be used for site-directed therapies, is still a matter of discussion.

Until recently most CUP patients were treated with empiric broad-spectrum chemotherapeutic regimes, resulting in median survival times of only 9 months. Only a subset of patients received a relatively-site-specific therapy, based on the presence of certain clinical or pathological features which prolonged the survival times.<sup>81</sup>

To determine the tissue of origin in CUP, clinical and pathological data as well as results from advanced imaging technologies are integrated and in most instances are sufficient. In cases where this procedure is not successful molecular assays can be of help in order to provide the patient with a site-specific therapy. At present three multi-marker profiling assays for the determination of the tissue of origin in CUP are commercially available, all of which are suitable for formalin-fixed tissue and only require little tumor tissue. The CancerType ID Assay (Biotheranostics, CA, USA) analyses the expression levels of 87 candidate and 5 house-keeping genes by qPCR.<sup>82</sup> The gene expression signature is then compared against a proprietary database consisting of 30 tumor types (54 subtypes). The Tissue of Origin Test (Response Genetics) is a microarray-based messenger RNA profiling test.<sup>83</sup> The expression levels of 2000 genes are measured and a similarity score in comparison to a database of 15 tumor classes (58 types/subtypes) is constructed. The Cancer Origin Test (Rosetta

**Table 1.** Overview of molecular tests with therapeutic implications recommended for routine testing in different tumor types (selection)

Tumor type	Affected gene(s)	Type of alteration	Method for detection	Related treatment
Breast cancer	HER2 PIKCA	Amplification SNV	IHC, ISH, Sequencing	Herceptin Reduced response to anti-HER2 treatment, in particular double blockade (Trastuzumab / Lapatinib) Prognostic assay (endocrine Tx vs Chemoendokrine Tx)
Colorectal cancer	Gene expression assays (EndoPre-dict, OncotypeDX etc.) RAS (KRAS, NRAS)	mRNA levels	qRT-PCR	Cetuximab / Panitumumab Imatinib, Sunitinib Imatinib, Sunitinib
GIST	KIT PDGFR	SNV SNV, indel	Sequencing	Vemurafenib, Dabrafenib, Trametinib
Malignant melanoma	BRAF	SNV	Sequencing	Sunitinib, Dasatinib, Imatinib
NSCLC	KIT EGFR	SNV, indel SNV, MNV, indel	Sequencing	Gefitinib, Erlotinib, Afatinib, Dacomitinib
	ALK	Translocation	ISH, IHC, Sequencing	Crizotinib, Ceritinib, Alectinib
	ROS1	Translocation	ISH, Sequencing	Crizotinib
	MET	Amplification	ISH, Sequencing	Resistance to EGFR TKIs
Serous ovarian cancer	BRCA1/BRCA2	SNV, MNV, indel	Sequencing	Olaparib
Walden-strom's, ABC-DLBCL	MYD88	Mutation L265P and others	Sequencing	IRAK174 inhibitors
Walden-strom's	CXCR4	Mutation truncating C-terminal region	Sequencing	CXCR4 inhibitors,(plerixafor), blocking antibodies
Classical hairy cell leukemia	BRAF	V600E mutation	Sequencing	Vemurafenib
Burkitt lymphoma, DLBCL, transformed FL	MYC	Translocation, amplification, overexpression	IHC for MYC protein, FISH	BET inhibitors, Protein translation inhibitors
DLBCL, FL, MCL, SLL/CLL	BCL2	Translocation, amplification, overexpression	IHC for BCL2 protein, FISH	BH3 mimetics

Abbreviations: ABC, activated B-cell; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; GCB, germinal center B-cell, IHC, immunohistochemistry, indel-insertion or deletion; ISH, *In situ* hybridization; MCL, mantle cell lymphoma; MNV, multiple nucleotide variation; PCR, Polymerase chain reaction; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia; SNV, single nucleotide variation; TKI, Tyrosine Kinase Inhibitor.

Genomics) profiles the expression of 64 micro RNAs by microarray and enables the identifications of 42 tumor types.<sup>84</sup> The accuracy of the assays has been evaluated in retrospective studies where the primary tumor was found later on and demonstrated good concordance.<sup>85</sup> Also, when comparing the gene expression assays with immunohistochemistry the performance was similar, with an advantage of the gene expression assays in tumors with poor differentiation.<sup>86,87</sup> Based on the available studies and a meta-analysis all assays seem to perform equally well. Therefore the decision if and what molecular test to use should be made for each case dependent on the clarity of the immunohistological results, availability of tissue, the tumor subtypes available for comparison in the respective test databases or access to a certain test. Furthermore it should be kept in mind that a clear clinical benefit has not been shown (and is difficult to demonstrate) for the molecular tests, due to a lack of suitable prospective studies.

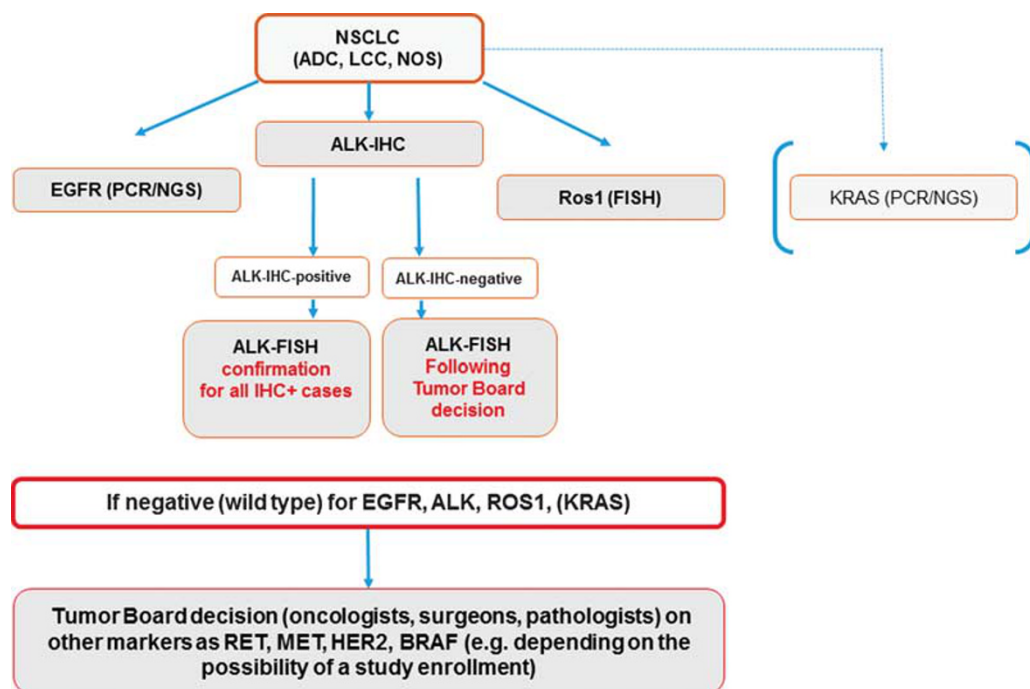
Chances for an improved treatment of CUP patients also lie in the use of next-generation sequencing, either as whole genome, exome or panel sequencing. Using these approaches common underlying molecular changes can be identified and, if druggable gene mutations are present, used for the application of targeted therapies. The use of the tumor-specific mutation pattern as the primary criteria for the choice of therapy, irrespective of the tumor origin, has been demonstrated in small scale studies<sup>88</sup> and will be used in so called basket studies, like e.g. the NCI MATCH trial starting in the middle of 2015.

### Lymphoid malignancies

The main effort in research on lymphoid malignancies during the last 30 years has been directed towards a precise, reproducible and world wide accepted classification that has ultimately led to the identification of nearly 110 different variants. This approach has had however only minimal influence on treatment that still heavily relies on the empiric administration of combination chemotherapy. The only major therapeutic change has been the incorporation of the anti-CD20 monoclonal antibody rituximab that can be considered as the first biomarker-based treatment for B-cell malignancies. The demonstration of CD20 expression on the neoplastic cells is a prerequisite for Rituximab application but it cannot predict response to treatment. The comprehensive assessment of genetics by means of whole-exome and whole-genome sequencing has revealed that genomic alterations are enriched within and across lymphoma variants. In addition, these studies have revealed that each individual lymphoma harbors a unique combination of genomic alterations. Therefore the major task of future lymphoma research will be the determination of key driver mutations and of possible oncogenic combinations of mutations that can serve as predictive biomarkers beyond the current classification. Some currently used predictive biomarkers are listed on Table 1 and discussed below.

Diffuse large B-cell lymphoma represents the most common subtype of Non-Hodgkin lymphoma world wide accounting for 30–40% of all newly diagnosed cases. It represents an aggressive neoplasm that is fatal without treatment. Current immunochemotherapy employing rituximab and a combination of cyclophosphamide, doxorubicin, vincristine and prednisolon (R-CHOP) can cure the majority of DLBCL patients, but most patients who fail R-CHOP will ultimately die from their disease. Molecular studies have elucidated a complex biologic heterogeneity of DLBCL. In particular, determination of the cell of origin can deliver important predictive information with DLBCLs of the activated B-cell (ABC) molecular subtype exhibiting an inferior outcome after R-CHOP treatment than DLBCLs of the germinal center cell (GCB) subtype (3-year progression-free survival 40% Vs 75%).<sup>89,90</sup> Unfortunately, there is currently not a standardized commercially available test that allows the precise determination of the molecular DLBCL subtype. Gene expression profiling represents the gold standard





**Figure 4.** NSCLC molecular testing algorithm at the Charité University Hospital (ADC: adenocarcinoma, LCC: large cell carcinoma, NOS: not other specified).

but it is not routinely available, as it requires fresh-frozen tissue specimens. In order to cope with paraffin-embedded tissue specimens, several immunohistochemistry-based algorithms have been established that are in fact an imperfect substitution for gene expression profiling and also suffer from the poor reproducibility of immunohistochemistry.<sup>91</sup>

Besides determination of the molecular cell of origin, other established biologic predictive factors that can influence therapy of DLBCL are MYC and BCL2. Rearrangements of MYC oncogene can be identified in approximately 10% of patients with DLBCL. The resulting increased expression of MYC protein is associated with increased proliferation of neoplastic cells and several studies could demonstrate that MYC rearrangement is associated with a poorer outcome in R-CHOP-treated DLBCL patients.<sup>92,93</sup> In addition, recent studies have shown that the impact of MYC is heavily influenced by BCL2. So called “double hit” lymphomas harboring MYC and BCL2 translocation represent a small group (approximately 5% of DLBCL cases) that are usually refractory to treatment with a median survival of approximately 8 months.<sup>94</sup> In addition, overexpression of MYC protein due to an up-regulation by other mechanisms than gene translocation can be detected by immunohistochemistry in up to 30% of DLBL patients. Interestingly a negative prognostic impact of such MYC protein overexpression is observed only in patients who simultaneously overexpress BCL2 protein. Such “dual expressers” account for approximately 25% of DLBCL patients who have a significantly poorer outcome than patients who express only one or neither protein.<sup>95–97</sup> It is thus conceivable that all DLBCL cases should be routinely assessed for translocations and protein overexpression of MYC and BCL2 in order to identify patients that could be subjected to alternative therapies.

Hairy cell leukemia (HCL) is an uncommon malignancy of mature B cells with a characteristic morphology and immunophenotype primarily affecting adult males. HCL is characterized by the involvement of the red pulp of the spleen, sinusoids of the liver, bone marrow, and peripheral blood ad extremely rarely by a lymphadenopathy.<sup>98</sup> Apart from the classical HCL (cHCL) that shows expression of CD20, CD22, CD25, Annexin A1, DBA.44,

tartrate-resistant acid phosphatase (TRAP), CD103, CD123 and T-bet two other subsets exist: the HCL variant (HCLv) with a different immunophenotype than cHCL with a lack of CD25, annexin A1, and/or TRAP expression,<sup>99</sup> and the HCL expressing the immunoglobulin VH4-34 rearrangement.<sup>100</sup> Identification of these variants is of importance as most patients with cHCL show an excellent prognosis with the treatment with cladribine or pentostatin with or without an anti-CD20 monoclonal antibody, while HCLv and VH4-34 HCL have a relatively poor response to this treatment and are associated with a shorter overall survival. The discovery of BRAF V600E mutation in the tumor cells of patients with hairy cell leukemia (HCL)<sup>101</sup> revealed that this represents not only a diagnostic but also a prognostic biomarker as it can differentiate between cHCL constantly harboring this mutation, while HCLv and VH4-34 HCL are BRAF mutation negative.<sup>102</sup> In addition, detection of BRAF mutation can also serve as a predictive biomarker in cHCL: although the majority of these patients show an excellent response to the mentioned therapy with purine analogs, up to 40% may have a relapse. As the treatment of the relapse can be difficult by decreased responsiveness to chemotherapy, progressive cytopenia and opportunistic infections other therapeutic options are discussed in the literature. Few case reports have convincingly shown that vemurafenib (an oral inhibitor of BRAF serine threonine kinase) is a promising option for patients with cHCL.<sup>103</sup>

Waldenström macroglobulinemia (WM) is an incurable B-cell neoplasm that belongs to the entity of lymphoplasmocytic lymphoma (LPL) and is defined as LPL with bone marrow involvement and an IgM monoclonal gammopathy of any concentration (WHO).

Using whole-genome sequencing two activating somatic mutations could be discovered in WM namely one affecting the Toll-like receptor and the other the CXCR4 receptor signaling.<sup>104</sup> Most of the patients with WM have a mutation L265P in MYD88 gene, while 1/3 of the patients show a mutation in the C terminus of CXCR4. The MYD88 mutation triggers the interleukin-1 receptor associated kinase (IRAK) and the Bruton’s tyrosine kinase (BTK) resulting in activation of the malignant cell growth, while 2 classes

of CXCR4 have been described: a nonsense (ns) and a frameshift (fs) mutation. A recent study could delineate the prognostic value of these mutations: WM patients with MYD88 L265P and CXCR4 ns mutations were overrepresented in the group of aggressive cases presenting with higher bone marrow disease involvement, higher IgM levels and hyperviscosity syndrome, while patients with MYD88 and CXCR4 wild-type presented with the lowest bone marrow involvement. Patients with MYD88 wild-type showed significantly higher mortality than patients with MYD88 L265P. Therefore determination of MYD88 and CXCR4 mutation status can be also of predictive value in the future as targeted therapies are going to be developed for WM patients.

#### Mesenchymal tumors

Within the heterogeneous group of human soft tissue tumors, GISTs are the most common mesenchymal tumors in the gastrointestinal tract. Discovery of KIT receptor tyrosine kinase expression and KIT mutations opened up novel therapeutic options. Approximately 85% of GISTs harbor a KIT mutation or a rare PDGFR- (platelet-derived growth factor) receptor alpha mutation.<sup>105</sup> Oncogenic KIT mutations occur within different domains and the location is relevant for sensitivity to targeted inhibitors, and in some cases for prognosis.<sup>106</sup> Most KIT mutations are found in the juxta-membrane domain encoded by exon 11, PDGFR alpha mutations are often D842V substitutions within the PDGFR alpha tyrosine kinase 2 domain encoded by exon 18.<sup>106</sup> Less than 1% of GIST cases feature BRAF V600E mutations,<sup>107</sup> and around 10% of adult GIST and most GISTs in children show none of the above mentioned mutations.<sup>108</sup> Some studies analyzed the different mutation types of GISTs that occur in patients with special syndromes, e.g. neurofibromatosis type 1 comparing to sporadic GISTs.<sup>109</sup>

Nowadays Imatinib-based therapy is recommended as a first-line treatment of patients with non-resectable and/or metastatic GIST as well as for adjuvant therapy in high risk patients.<sup>98,110–112</sup> Tumors with a KIT mutation in exon 11 are most sensitive to Imatinib, while GISTs with a PDGFRA mutation in exon 18 (D842V) seem to be resistant to this therapy.<sup>113,114</sup> GISTs with a KIT mutation in exon 9 need a higher dose of Imatinib (> 400 mg/day) to gain a longer progression-free survival.<sup>115</sup> Furthermore, some patients develop a secondary resistance under Imatinib therapy. Sunitinib malate as a multi-targeted tyrosine kinase inhibitor has been approved for second-line therapy in these patients with disease progression under Imatinib therapy.<sup>116,117</sup>

Within the last two years genetic landscapes determined via whole-genome or exome sequencing of other childhood mesenchymal tumors including Ewing Sarcoma (ES) and Rhabdomyosarcoma (RMS;<sup>118</sup>) have been published. Shern *et al.* unraveled an extensive genetic alteration map for Rhabdomyosarcoma. Their data indicate that in addition to PAX3/7 fusions also other genetic alterations including well-known oncogenes such as NRAS, KRAS and FGFR can occur in a subgroup of RMS patients, albeit with low incidence. The observation that the RTK/RAS/PIK3CA axis is affected in a large proportion of patients should be considered in more detail and included in novel clinical trials. Current efforts treating the PAX-fusion target IGF1R in RMS patients with the anti-IGF1R antibody either in combination with chemotherapy or other signaling pathway inhibitors such as temsirolimus have not proven satisfying results and lack a stringent biomarker support.<sup>119,120</sup> Genomic landscapes of Ewing sarcoma were published by several groups via whole-genome/exome or targeted sequencing,<sup>121,122</sup> see also review by Sand *et al.*<sup>123</sup>). In general, relatively low overall numbers of genetic alterations such as CNVs, indels and other structural variants not being EWSR1-ETS were discovered. Among the most frequent alterations were mutations within the STAG2, CDKN2A and TP53 genes. Interesting, mutational inactivation or loss of STAG2 alone

or together with TP53 alterations seems to be a prognostic marker as it is frequently associated with disease dissemination and poor outcome of the patients.<sup>121</sup> More recently, Agelopoulos *et al.*<sup>124</sup> confirmed these results but in addition discovered higher numbers of mutations in relapsed tumors and copy number gain of the FGFR1 gene in roughly one-third of the patients. Preclinical investigations using an FGFR1 inhibitor showed promising results and might lead to improved clinical trials and biomarker development for Ewing sarcoma in the near future.

In addition to RMS and ES, also osteosarcomas have been analyzed in detail via exome sequencing.<sup>125</sup> This analysis revealed an interesting pattern of 'kataegis' (localized hypermutations) in half of the samples tested, structural alterations and copy number alterations, yet rather few targetable mutations. The most frequently altered genes comprise the TP53, RB1, ATRX and DLG2 genes. While no structural alterations were determined via sequencing in the EGFR family, clinical trials are already under way testing the efficacy of both kinase inhibitors and antibodies against members of the EGFR family in osteosarcomas and other mesenchymal cancers such as synovial sarcomas (see review by Wang *et al.*<sup>126</sup>) These approaches are based on observations that sarcomas can overexpress EGFR and other members of this RTK family, however the results rather dampen the expectations and await further detailed investigation.

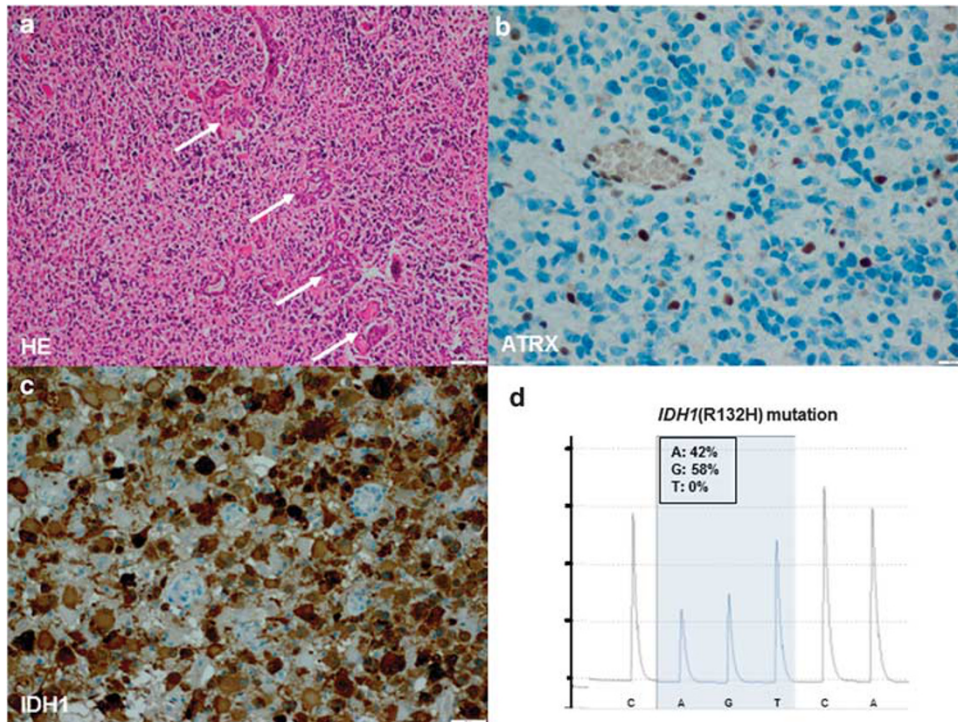
#### Central nervous system tumors

Depending on certain cytological and histological criteria the World Health Organization (WHO) assigns four grades for CNS tumors (WHO grade I to IV). While these grades are mainly based on histopathological criteria characterizing malignancy, they also include and thus predict the expected clinical course of the patient.<sup>127</sup>

Based on the rapid progress in the molecular understanding of the pathogenesis of brain tumors, in particular of glial tumors, new molecular signatures have been identified, which serve as reliable diagnostic, prognostic and predictive molecular markers (Figure 5). Based on several clinical studies three molecular markers—namely, 1p/19q co-deletion, MGMT promoter methylation and mutations in the IDH1/2 genes—just to mention the to date most prominent ones, were routinely used to improve and manage patients' outcome.

**Combined loss of 1p/19q in glial tumors.** It has been shown that about 80% of oligodendroglioma (grade II), 60% of anaplastic oligodendroglioma (grade III) and 50% of mixed glioma are characterized by combined allelic deletions on chromosome 1p and 19q.<sup>85</sup> Two clinical studies demonstrated that patients with anaplastic glioma benefit from a combined radio- plus PCV chemotherapy if the tumor harbor a combined 1p/19q loss.<sup>128,129</sup> **MGMT promoter methylation:** The O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) is a DNA repair enzyme that removes the alkylation of the O<sup>6</sup> position of guanine, which protects DNA alkylation and finally apoptosis. Methylation of the MGMT promoter results in gene silencing due to reduced proficiency to repair DNA damage induced by alkylating chemotherapeutic agents like temozolomide. MGMT promoter methylation occurs in about 40% of primary glioblastoma (WHO grade IV) and is associated with an increased survival after radiotherapy and chemotherapy with temozolomide.<sup>130,131</sup> Recently, two independent multicentric studies demonstrated the predictive relevance of MGMT status in older glioblastoma patients (> 65 years,) which benefit from a temozolomide therapy alone if MGMT is methylated.

**Mutations of IDH1 and IDH2.** IDH (Isocitrate-Dehydrogenase)1 and its mitochondrial isoform IDH2 encode for proteins which catalyze isocitrate to  $\alpha$ -ketoglutarate and play important roles in the



**Figure 5.** (a) malignant glioma tumor with numerous vascular proliferations (Glioblastoma WHO grade IV), (b) the tumor cells show a nuclear loss of the Atrx Protein according to a mutation in the transcription factor ATRX, which is a molecular feature of astroglial differentiation. Endothelial cells and reactive astrocytes are positive. (c) the gemistocytic tumor cells strongly react with antibodies against the mutation specific marker IDH1 indicating an IDH1(R132H) mutation. IDH mutant malignant glioma show longer overall survival than those with IDH wild-type neoplasms. (d) IDH1(R132H) mutation was confirmed by pyro-sequencing.

cellular control of oxidative processes. *IDH1/2* mutations functionally results in global changes of the tumor-epigenome. The presence of somatic *IDH1/2* point mutations in the vast majority of low-grade glioma and secondary glioblastoma is helpful in the differentiation to primary glioblastoma, pilocytic astrocytoma and other primary brain tumors, which are not characterized by these mutations. The presence of *IDH1/2* mutations in anaplastic glioma and glioblastoma has also a prognostic relevance as patients with *IDH* mutant tumors show longer overall survival than those with *IDH* wild-type neoplasms.

The continuous identification of novel molecular signatures, apart from the above mentioned ones, forces state-of-the-art neuropathology to broaden its diagnostic molecular spectrum by persistently implementing such molecular markers within the standard repertoire of diagnostic procedures used to characterize brain tumors.

In current meetings concerning the revision of the fourth edition of the WHO classification of central nervous system tumors it has been discussed if for some entities (in particular oligodendroglial and some pediatric nervous system tumors), molecular information are required to provide an 'integrated' diagnosis and only an descriptive histological diagnosis is acceptable if no molecular diagnostic testing is available. For instance in case of mixed glioma (oligoastrocytoma), independent of the histological phenotype the *IDH1/2*, *ATRX* and *1p/19q* status will define the overall diagnosis.<sup>132</sup>

## CONCLUSIONS

In addition to what is written in the previous paper<sup>1</sup> we can state that the development of targeted therapy is dramatic, the number of new drugs in clinical studies is enormous and the resulting challenges for predictive pathology/companion diagnostic are

indeed substantial. Since currently the vast majority of the assays are tissue-based the responsibility of accurate performance lies in the hand of pathologists. The scientific societies have to be alert to cover this chance. Education of doctors and technicians, quality control of technical procedures and the intellectual interpretation of the results are crucial to provide reliable results. Clearly this will play an increasing role in the future structure of tissue-based diagnostics.

The multilayer analyses of malignant tumors produce an increasing number of data creating a high complexity of information for more or less each tumor. To manage this situation interdisciplinary becomes an absolute important prerequisite in cancer treatment. This is true in daily work and in scientific projects. A consequence of this situation is the necessity to build up comprehensive cancer centers which can provide the broad spectrum of knowledge and experience.

In addition an up-dated design of clinical studies is needed to bring the possible advantages of the new multiplex approach to patients. Here all medical disciplines, the regulatory authorities and pharma industry have to stay and think together how the progress can be translated fast and safe into the clinic. This may become a severe challenge in particular for rare tumor types.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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