Assigning evidence to actionability: An introduction to variant interpretation in precision cancer medicine

Peter Horak1,2,3, Jonas Leichsenring4,5, Hannah Goldschmid5, Simon Kreutzfeldt1,2, Daniel Kazdal5,6, Veronica Teleanu1,2,7, Volker Endris5, Laura Gieldon8, Michael Allgäuer5, Anna-Lena Volckmar5, Nicola Dikow3, Marcus Renner1,2, Martina Kirchner5, Roland Penzel5, Carolin Ploeger3,5, Regine Brandt5, Huriye Seker-Cin5, Jan Budczies2,5,6, Christoph E. Heilig1,2, Olaf Neumann5, Christian P. Schaaf8, Peter Schirmacher2,3,5, Stefan Fröhling1,2,3, Albrecht Stenzinger2,3,5,6

1Department of Translational Medical Oncology, National Center for Tumor Diseases (NCT) Heidelberg and German Cancer Research Center (DKFZ), Heidelberg, Germany
2German Cancer Consortium (DKTK), Core Center Heidelberg, Heidelberg, Germany
3Center for Personalized Medicine (ZPM), Heidelberg, Germany
4Institut für Pathologie, Zytologie und molekulare Diagnostik, Regiomed Klinikum Coburg, Coburg, Germany
5Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany
6German Center for Lung Research (DZL), Translational Lung Research Center Heidelberg (TLRC-H), Heidelberg, Germany
7Department of Internal Medicine V, Heidelberg University Hospital, Heidelberg, Germany
8Institute of Human Genetics, Heidelberg University, Heidelberg, Germany

Correspondence
Peter Horak, Department of Translational Medical Oncology, NCT Heidelberg, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany.
Email: peter.horak@nct-heidelberg.de

Abstract
Modern concepts in precision cancer medicine are based on increasingly complex genomic analyses and require standardized criteria for the functional evaluation and reporting of detected genomic alterations in order to assess their clinical relevance. In this article, we propose and address the necessary steps in systematic variant evaluation consisting of bioinformatic analysis, functional annotation and clinical interpretation, focusing on the latter two aspects. We discuss the role and clinical application of current variant classification systems and point out their scope and limitations. Finally, we highlight the significance of the molecular tumor board as a platform for clinical decision-making based on genomic analyses.

KEYWORDS
molecular biomarker, molecular tumor board, precision oncology, variant classification
1 | PRECISION CANCER MEDICINE AND INCREASING DATA COMPLEXITY

Since cancer is a genetic disease, analysis of a tumor’s genetic material with regard to sequence variants and structural alterations using next-generation sequencing (NGS) is playing a crucial role in modern oncology and pathology. Additional layers of high-throughput molecular information, in the form of proteomic, methylation, and transcriptomic data obtained from tumors or liquid biopsies, are increasingly used for diagnostic, prognostic, or predictive purposes in cancer patients. Broad molecular characterization of cancer samples lays the foundation for personalized precision medicine, leading to a better understanding of tumor biology, and identification of targetable genetic alterations, thus improving patient outcomes. The fact that an increasing number of molecular biomarker (MB)-stratified clinical trials led to the approval of new cancer therapies supports this approach to cancer medicine.

Molecular biomarkers can consist of either therapeutic target structures that can be directly inhibited by specific drugs, indirect indicators of drug response, or molecular “aggregate states” (e.g., high-level microsatellite instability [MSI-H] or the deficiency of homologous recombination repair [HRD]), which indicate a therapeutically exploitable vulnerability of the tumor. The latter belong to the group of complex predictive biomarkers, defined by the necessity to assess more than a single genetic alteration (e.g., single nucleotide variant [SNV], small insertion or deletion [indel] or a somatic copy number alteration [scNA]) and/or are based on broad molecular profiling of tumors. With the increasing size of NGS-based panels and a transition to whole-exome or whole-genome sequencing (WES/WGS) of tumors, these complex biomarkers gain accessibility and relevance. In addition, several biomarkers can be combined to form a composite score with higher specificity and sensitivity to predict response or resistance to a specific cancer therapy. The genetic and molecular context of a tumor entity has a significant modulatory influence on the prognostic and predictive value of MBs and has to be accounted for. These newly developed complex and composite MBs need to be carefully evaluated and benchmarked in a clinical setting before they can be implemented in routine clinical practice.

WES and WGS are currently carried out predominantly in advanced stages of disease and in the context of precision oncology programs and prospective trials. They inevitably lead to the discovery of numerous genetic alterations with the ensuing challenge to attribute them a biological and clinical relevance. While the clinical relevance for some MBs can be assessed quite easily with data from clinical studies restricted to one tumor entity (e.g., EGFR L858R in the context of a stage IV adenocarcinoma of the lung), the clinical relevance of the same mutation in a different tumor context (EGFR L858R in gastric adenocarcinoma) is less clear. This also applies, for example, to alterations of genes (beyond BRCA1 and BRCA2) that are involved in homologous recombination DNA repair and can indicate responsiveness to poly(ADP-ribose) polymerase (PARP) inhibitors or platinum-based chemotherapies. Increased reporting of such alterations due to broad molecular profiling of different cancer entities led to the development of several classification systems for assessing evidence and defining clinical relevance of MBs in cancer.

Challenges can also arise when a molecular analysis detects multiple therapeutic targets and MB-drug associations with different levels of evidence. Currently, molecular tumor boards (MTB) have an important role in such scenarios in order to evaluate relevance and possible interactions between individual MBs. They can also put the molecular profile in the context of the specific disease and consider additional clinical factors. For MTB to work successfully, its members need to establish a harmonized approach for evaluating molecular alterations based upon a jointly agreed standard. While this overview is not intended to replace experience and self-study, we hope to provide interested readers from various fields (pathology, oncology, bioinformatics, and other involved disciplines) orientation in this quickly evolving field and equip them with a short guide for standardized assessment of MBs.

2 | STEP 1: IDENTIFICATION OF TUMOR-SPECIFIC AND GERMLINE VARIANTS AND BIOMARKERS

The identification of genetic variants and MBs obtained from NGS-based analyses is now a fairly well standardized process for which a number of open source and commercial bioinformatic algorithms and corresponding software solutions are available. These have their individual strengths and weaknesses, but generate comparable results providing an overall robust basis for further analysis. This is followed by the primary annotation using bioinformatic tools and querying sequence and variant databases. This process leads to annotation of each variant with a set of metadata, such as the position of the variant in relation to the gene or the genetic region, the predicted cDNA and amino acid sequence (Human Genome Variation Society [HGVS] nomenclature) and its prevalence in different populations and variant databases. Although standardization of this step is feasible, the databases in use differ widely between institutions. This part of analysis should include technical quality filtering of variants for further classification and interpretation. It should not provide filtering with regard to functional or clinical relevance, nor any prioritization of the variants. In “tumor-only” sequencing (i.e., without parallel analysis of a germline sample), filtering data to eliminate common single nucleotide polymorphisms is also a task of the primary annotation. In WES and WGS, germline and somatic data are generated directly.

3 | GERMLINE ANALYSIS AND GENETIC COUNSELLING

A significant fraction (9%–12%) of cancer patients carries a germline mutation predisposing for different hereditary cancer syndromes and a majority of patients expresses a clear preference to be informed about the results of germline analyses. It is thus necessary to consider possible germline involvement of (likely) pathogenic variants that
Somatic and germline variants cannot be discriminated by “tumor-only” sequencing and parallel tumor and germline sequencing should be attempted whenever possible. Family history, results of previous germline analyses of the patient or patient’s family, tumor biology (including variant allele frequency [VAF] in relation to tumor cell content), and patient’s phenotype can all be indicators of putative germline involvement. Interpretation of possible germline variants needs to be performed by a clinical geneticist and based on the aforementioned clinical and biological information. Patients need be informed about the possibility of incidental findings and informed consent should be obtained before performing the analysis, thus minimizing the risk of confronting the patient with unsolicited results. Variant interpretation in a germline context should be performed in accordance with published guidelines, such as the American College of Medical Genetics and Genomics (ACMG) criteria for germline variant interpretation.29 Sequential germline analysis should be offered to patients with possible tumor predisposition identified by “tumor-only” sequencing. Selection of genes with a significant probability of clinically relevant germline involvement—necessitating reporting and follow-up analysis—should be made carefully, updated regularly, and guided by published recommendations.30 Variants of unknown significance should not be followed up in the germline. Follow-up germline analyses require additional genetic counseling and patient consent.

4 | STEP 2: ASSESSING BIOLOGICAL AND FUNCTIONAL IMPACT OF IDENTIFIED BIOMARKERS

The second step leads to a functional and biological annotation of individual somatic variants/MBs. To this end, curators obtain information about a variant’s effect on gene and protein function and its role within a molecular signaling pathway and the specific disease. This annotation may be facilitated by the use of additional public or commercial datasets, which are derived from tumor-specific population databases (e.g., COSMIC, Cancer Hotspots, cBioPortal), in-silico predictors (e.g., FATHMM, CADD) and also literature-based, manually curated databases (e.g., OnkoKB, CIVIC, JAX-CKB).31 Automated annotation using these databases is hampered by historical inconsistencies in gene names and transcripts, inconsistent application of existing standards as well as non-HGVS annotation used by some bioinformatic tools.32,33 In addition, the databases have to be maintained and versioned in order to be used for automated search and reporting. Therefore, additional manual search of the published biomedical literature is essential and yields important information beyond that obtained from search algorithms and databases in a majority of cases. This task is currently highly reliant on human resources (Figure 1) and needs to be standardized and harmonized across and within institutions. The heterogeneity of implemented workflows for functional annotation of cancer variants/MBs and assessments of variant oncogenicity have an immediate impact on clinical interpretation and clinical decision-making in the MTB.

Although the functional role of a variant is closely linked to its clinical (therapeutic) relevance, we consider the assessment of a biological effect of a variant/MB on protein function (oncogenicity) an independent step (Figure 1). Despite significant technological advances, there is still great variability in the functional and, as a consequence, also the clinical classification of somatic variants in tumors. Guidelines for variant classification published by European and American professional societies evaluate the clinical relevance of somatic variants in tumors, address the issue of standardizing functional annotation, and recommend datasets or databases that are to be considered to determine the oncogenicity of a variant, but they stop short of devising a formal model for their use. To enable this standardization, a guideline for the interpretation of the oncogenicity of somatic variants in tumors is being developed by the Variant Interpretation for Cancer Consortium (VICC, www.cancervariants.org). This guideline might be considered the first step towards a harmonized interpretation of somatic genetic variants in the context of malignant diseases and will make a significant contribution to the assessment of their therapeutic relevance and aid future classification of evidence. Since characterization of germline variants by ACMG/Association for Molecular Pathology (AMP) variant classification guidelines29 addresses the risk of phenotypically healthy carriers of a genetic trait, it requires a very high probability for the assessment of a possible
pathogenicity (with more than 90% certainty), as further screening, preventive measures, and in some cases, prenatal diagnostics, are based on it.\textsuperscript{29} On contrary, oncogenicity assessment of somatic variants might tolerate a more progressive approach. Its aim should be the selection of variants effecting gene or protein function with a sufficiently high probability to inform the therapeutic decision-making process for cancer patients whose tumors are profiled in advanced stages of their disease. This standardized evaluation then enables functional annotation and clinical interpretation using the published classification systems.

Assessing clinical relevance strongly relies on published clinicogenomic datasets that often provide an inaccurate genetic profile of a tumor entity with missing data on rare variants. This might be best illustrated by the various classes of BRAF point mutations and associated clinical datasets. The most common V600 mutations belong to the BRAF class I mutations and induce a RAS-independent, constitutively active BRAF kinase, which is able to activate the signaling pathway as a monomer. Class I mutations can be targeted therapeutically using BRAF-inhibitors, which is the accepted standard of care for several cancer entities based on robust clinical data stemming from randomized phase 3 trials. Class II mutations (e.g., K601E, L597Q, and G469A) signal as constitutively activated mutant dimers. BRAF monomer inhibitors, such as vemurafenib, are less effective in inhibiting protein function of these mutants. Data for (B)RAF-inhibitor or MEK-inhibitor efficacy in tumors with class II mutations is mostly preclinical or coming from individual case reports. In contrast, class III mutations (e.g., D594G or G466V) show impaired kinase activity. They lead to dimerization between wild-type BRAF and CRAF, induce allosteric CRAF activation,\textsuperscript{34} and thus make BRAF-directed therapy obsolete. Nevertheless, CRAF-inhibitors or MEK-inhibitors might be effective in these cases albeit robust clinical evidence is missing.\textsuperscript{35} All three classes of BRAF variants are oncogenic and might represent an actionable target, but only BRAF V600 mutations have a validated clinical role. Functional classification of a MB has direct implications for the therapeutic decision, but the assessment of its oncogenicity is independent from published data on sensitivity to a specific inhibitor, that is, actionability and thus a more stable feature of a the MB. Therefore, we believe that functional annotation should be the first step of the clinical evaluation process, leading to structured interpretation of somatic molecular alterations in the MTB (Figure 1).

5 | \textbf{STEP 3: EVALUATING CLINICAL EVIDENCE OF BIOMARKER-DRUG ASSOCIATIONS}

Identification and categorization of oncogenic MBs, as described in the previous section, is a prerequisite for further interpretation, but not sufficient to judge the potential therapeutic benefit. We focus here on selected aspects influencing the treatment decision, including the properties of a MB, quality and availability of precision oncology knowledge databases, evidence classification frameworks, and the integration of molecular data with additional clinical parameters.

5.1 | Properties of the MB

In addition to oncogenicity as discussed, evaluation of the MB as predictive includes the mechanistic relationship between the biomarker present and the possible therapy: (i) direct inhibition (BRAF-inhibitor for BRAF mutated tumors), (ii) pathway inhibition (MEK-inhibitor for BRAF mutated tumors), (iii) synthetic lethality (PARP-inhibitor for BRCA1/2 mutated tumors), and (iv) correlative relationships with unexplained biological mechanisms. In case of SNVs and indels, quantitative analysis of MB traits is reduced to the evaluation of allele frequencies in relation to the tumor cell content and possible intratumoral heterogeneity. In more complex MB scenarios (tumor mutational burden [TMB], HRD score), a specific threshold for functional relevance and therapeutic accessibility has to be defined and validated for each MB, method and tumor entity. To benchmark and cross-evaluate MB thresholds using different methodologies and bioinformatic pipelines will be a challenging task on its own.

Considering the histopathological context of a MB-drug association is another important component of clinical evaluation. Presence of (unspecified) resistance mechanisms, for example, leads to different therapeutic recommendations in BRAF V600 mutated colorectal carcinoma than in BRAF V600 mutated melanoma or NSCLC. Association between the MB and a histopathological entity might be considered in the oncogenicity assessment of rare variants, for example, potentially higher likelihood of oncogenicity of rare BRAF variants in tumor entities that are associated with known driving oncogenic BRAF mutations.\textsuperscript{36} The decision-making process also incorporates the possible synergistic effects of a compound with additional, molecularly targeted or conventional (radiation therapy, chemotherapy) treatment strategies, which might or might not be based on additional MBs. Another aspect concerns the modulating effect of additionally identified MB on the MB-drug association in question. This consideration can include a known, clinically validated resistance mutation, but it can also be based on several MB-drug associations in one tumor with discrepant data regarding their predictive effect. This evaluation is supported by current evidence and only insufficiently reflected in published evidence classification systems.

5.2 | Precision oncology databases

Therapeutically relevant MB-drug associations can, in part, be found using the same databases as those used for the assessment of MB oncogenicity in Step 2. Standardization and harmonization of these knowledge bases is an ongoing effort,\textsuperscript{33} resulting in the need for ongoing re-assessment of their content and making the automated extraction of MB-drug associations difficult. Manual curation and broad interdisciplinary expertise (molecular biology, bioinformatics, pathology, and medical oncology) are thus major requirements for the evaluation of this data. Final clinical decision about the relevance of a specific MB-drug association for a specific patient can only be made with detailed knowledge of the patient’s medical history, adverse
5.3 | Variant classification systems

Published evidence classification systems for clinical interpretation of somatic variants/MBs analyze and evaluate their diagnostic, prognostic and therapeutic implications. Established international classification systems proposed by professional societies are the “Joint Consensus Recommendation” (JCR) of the American Society of Clinical Oncology (ASCO), AMP, ACMG, and the College of American Pathologists (CAP) as well as the “ESMO Scale for Clinical Actionability of Molecular Targets” (ESCAT) of the European Society for Medical Oncology (ESMO). In addition, there are classification systems established at the national level. In Germany, the classification of the National Center for Tumor Diseases (NCT) and the German Consortium for Cancer Research (DKTK) is the most widely used. Some of these evidence classification systems are implemented in knowledge databases, clinical laboratories and commercial applications.

5.3.1 | Joint consensus recommendation

This classification system is based on clinical and preclinical evidence and is used to evaluate actionable ability of SNVs, indels, gene fusions, and sCNA, which is stratified to four tiers. Consideration of regulatory approval and classification of diagnostic and prognostic MBs are unique features of the JCR framework.

5.3.2 | ESMO scale for clinical actionability of molecular targets

In contrast to the JCR classification, the ESCAT classification is applicable regardless of the respective approval status. ESCAT focuses on clinical benefit and incorporates the ESMO Magnitude of Clinical Benefit Scale (MCBS 1.1), undertaking a complex evaluation of clinical trials. ESCAT also offers a unique level of evidence for in-silico data.

5.3.3 | National center for tumor diseases classification

The NCT classification used in the German networks of precision medicine focuses on three parameters for determining the relevance of an MB drug association: (i) tumor entity (i.e., histopathological classification), (ii) preclinical versus clinical evidence, and (iii) strength of clinical evidence (Table 1). NCT classification was developed for daily use in MTBs—requiring complexity reduction—and offers flexibility to adapt to the heterogeneous body of scientific literature.

6 | EXAMPLES OF BIOMARKER CLASSIFICATIONS

No evidence ranking system or decision tool can be used without a “healthy dose of judgment and thought,” and no evidence classification replaces the interdisciplinary discussion in the MTB. The harmonization of the therapeutic variant classification was made possible by the publication of the aforementioned guidelines, but due to their heterogeneity, complexity, and different weighing of the relevant variables, these guidelines have not been uniformly implemented yet. Application of the three variant classification systems with explanation of their differences is presented in the following examples (Table 2).

**Example 1:** The entity-agnostic approval of larotrectinib in patients harboring NTRK gene fusions was based on a combined analysis of three phase 1–2 basket trials. Due to the rarity of the entities included in these trials and the fusion itself, most histopathological entities were represented by only a handful of patients. The evidence can be clearly defined as I-C in the ESCAT system. The approval of the drug by the FDA leads to Tier I-A classification in JCR, but does not offer any further assessment based on the strength of clinical evidence (phase 1–2 basket trials) or entity. NCT classification allows m1A-Z classification of the published analysis, however, a more granular classification with regard to a specific histopathological entity can be used (i.e., m1C-Z or m2A-Z).

**Example 2:** Another clinically relevant constellation can be exemplified by BRAF V600 mutations and associated BRAF/MEK-inhibitor combination of dabrafenib and trametinib. The JCR classification (Tier I-A) for NSCLC with BRAF V600E is based on approval data from a non-randomized, open label, phase 2 study. The same evidence leads to an ESCAT I-B classification. The NCT classification is m1A-Z, as it does not differentiate between randomized and non-randomized prospective studies. Looking at the rare BRAF V600K variant in terms of its classification in NSCLC reveals surprising results: The V600K variant was not included in the approval study in NSCLC and since the FDA approval (in contrast to EMA) is specific for the V600E variant in NSCLC, only the ESMO guideline (biomarker included in professional guidelines) could lead to a Tier I-A2 JCR classification. ESCAT level falls to III-A due to the missing clinical data. In case data from other tumor entities are taken into account, ESCAT level rises to I-C again. The NCT classification is also lower here in the absence of prospective data for the entity and is based on data from studies in melanoma (m2A), but indicates the relevant approval of the EMA (m2A-Z). In melanoma, the same MB-drug association would be classified as JCR Tier I-A, ESCAT I-A, and NCT m1A-Z (Table 2).

Intricacies of the classification are further demonstrated by data on vemurafenib in BRAF V600 mutated NSCLC. Vemurafenib is neither approved by the FDA nor by the EMA for NSCLC, but clinical studies and individual case reports support the effectiveness of the BRAF-inhibitor in this setting. Here as well, a distinction must be made between the common BRAF V600E and other BRAF V600 mutations. With BRAF V600E, the JCR leads to a Tier I-B recommendation (expert consensus without FDA approval). Evidence level I-C
TABLE 1  NCT level of evidence classification

| Level of evidence | Basis for classification | Tissue context | Description of classification |
|-------------------|--------------------------|----------------|-------------------------------|
| m1A               | Clinical data            | Same histopathologic entity | The predictive value of the biomarker or clinical effectiveness of the corresponding drug in a molecularly stratified cohort was demonstrated in a prospective study or meta-analysis in the same tumor type. |
| m1B               | Clinical data            | Same histopathologic entity | The predictive value of the biomarker or clinical effectiveness of the drug in a molecularly stratified cohort was demonstrated in a retrospective cohort or case–control study in the same tumor type. |
| m1C               | Clinical data            | Same histopathologic entity | A case study or single unusual responder indicates that the biomarker is associated with response to the corresponding drug in the same tumor type. |
| m2A               | Clinical data            | Different histopathologic entity | The predictive value of the biomarker or clinical effectiveness of the corresponding drug in a molecularly stratified cohort was demonstrated in a prospective study or meta-analysis in a different tumor type. |
| m2B               | Clinical data            | Different histopathologic entity | The predictive value of the biomarker or clinical effectiveness of the drug in a molecularly stratified cohort was demonstrated in a retrospective cohort or case–control study in a different tumor type. |
| m2C               | Clinical data            | Different histopathologic entity | A case study or single unusual responder indicates that the biomarker is associated with response to the corresponding drug in a different tumor type. |
| m3                | Preclinical data         | Not applicable | Preclinical data demonstrate that the biomarker predicts response to a specific drug, supported by a scientific rationale. |
| m4                | Biological rationale     | Not applicable | A biological rationale exists that associates the biomarker with altered activity of cellular pathways/processes or drug sensitivity without direct clinical or preclinical evidence for a response to the drug. |

Additional modifiers

is  In situ data and studies on patient material (e.g., immunohistochemistry, fluorescence in situ hybridization) support the biomarker and the level of evidence.

iv  In vitro data support the biomarker and the level of evidence.

Z  Drug is approved for use with the specific biomarker.

R  Biomarker predicts resistance to the drug.

TABLE 2  Examples of divergent variant classifications based on JCR, ESCAT, and NCT classifications

| Molecular biomarker | Drug                  | Entity       | JCR     | ESCAT | NCT     |
|---------------------|-----------------------|--------------|---------|-------|---------|
| NTRK-Gene Fusions   | Larotrectinib         | Solid tumors | Tier I-A| I-C   | m1A-Z   |
|                     |                       |              |         |       | m1C-Z, m2A-Z |
| BRAF V600E          | Dabrafenib + Trametinib | NSCLC       | Tier I-A| I-B   | m1A-Z   |
| BRAF V600K          | Dabrafenib + Trametinib | NSCLC       | Tier I-A| III-A | m2A-Z   |
| BRAF V600K          | Dabrafenib + Trametinib | Melanoma    | Tier I-A| I-A   | m1A-Z   |
| BRAF V600E          | Vemurafenib           | NSCLC       | Tier I-B| I-C   | m1A     |
| BRAF V600K          | Vemurafenib           | NSCLC       | Tier II-C| II-B | m1A     |
|                     |                       |              |         | III-A | m1C     |
| TMB (≥10 mutations/MB) | Pembrolizumab         | Solid tumors | Tier I-A| I-C   | m1B-Z   |
|                     |                       |              |         | I-B   | m1A-Z   |
| HRD ≥42             | Olaparib + Bevacizumab | Ovarian Cancer | Tier I-A| I-A   | m1A-Z   |

Note: In italics—alternative classification.

based on a basket trial\textsuperscript{12} can be used for the ESCAT classification, since the only prospective study in this setting shows a low MCBS.\textsuperscript{43} As this study presents clinically relevant response rates without survival data, evaluation according to ESCAT also allows the II-B classification. Judging the same evidence coming from a prospective, open-label study leads to m1A classification by the NCT framework.
Due to its rarity, the BRAF V600K mutation was only recorded in three NSCLC patients in these studies and is not covered by the consensus statements of professional societies. This leads to a JCR Tier II-C assessment. The ESCAT evidence classification has a number of possible interpretations in this case: the studies with vemurafenib included BRAF V600 mutated tumors, but the exact number of BRAF V600K mutated cases is in the single-digit range.

The basket trial had 0/20 tumors with BRAF V600K, the prospective, open-label study 2/100, and 1 out of 35 NSCLC samples with BRAF V600K were present in the retrospective analysis. Treatment of two BRAF V600K NSCLC patients with vemurafenib was documented in the prospective, open-label study, with individual progression-free survival times of 2.1 and 6.8 months and partial remission in one of the two cases. This clinical trial could be evaluated by ESCAT as II-B (response without survival data), but whether this classification is justified due to the rarity of the BRAF V600K remains unclear. A more stringent interpretation would lead to ESCAT III-A, with evidence from other entities (e.g., BRAF V600K mutant melanoma). The NCT classification allows the m1A evaluation of the data as a prospective, open-label study with vemurafenib in BRAF V600 mutated tumors, but the evidence level can be adjusted to m1C due to the low number of enrolled patients with BRAF V600K mutations. Consideration of individual case reports of molecularly characterized tumors and assigning them a level of evidence is a distinctive feature of the NCT classification.

6.1 Gene fusions

Implementation of targeted RNA sequencing approaches in routine diagnostics allows detection of clinically relevant gene fusions in a time-efficient, cost-efficient, and tissue-efficient manner. Currently, two different assay designs are used in the clinical field. First, classic multiplex RT-PCR-based approaches, which target specific fusion transcripts with primers at each side of a known fusion breakpoint. These methods are limited by detecting fusions from a predesigned panel of target sequences only. Second, assays based on anchored multiplex PCR or assays using hybrid capture-based target enrichment—both depending on only one known fusion partner—are available and used for more comprehensive analyses addressing rare or yet unknown fusion variants. The increasing number and variety of novel fusions identified in cancer exceed the capacity of databases to profile them and provide detailed information regarding their functionality and actionability. One benefit of open assays is their ability to detect the diversity of loss-of-function fusions with oncogenic potential. This is the case for the complete loss of gene function, for example, in BRCA1/2 fusions, or for the loss of regulatory domains, as observed in FGFR2 fusions.

For example, loss of heterozygosity (LOH) as well as chromosomal rearrangements leading to an inactivating translocation of BRCA1 may render a patient eligible for PARP-inhibitor therapy. Currently, only few recurrent fusion partners in these loss-of-function fusions are described. The aforementioned examples are promiscuous towards their fusion partners, and many of detected fusions are thus unique individual events.

Additional clinically relevant information can be obtained from these RNA-based assays in the form of (oncogenic) isoform expression, for example, FGFR2, that might be targetable with specific inhibitors. This methodology also allows assessing RNA expression of transcripts from translocation events or from large deletions resulting in out-of-frame fusions that are most likely destined for nonsense-mediated mRNA decay and thus adds another level of complexity to the classification and interpretation of MBs.

A fusion or rearrangement of genetic regions is a complex event that brings about many pitfalls when it comes to proper annotation. Due to the bipartite or sometimes even tripartite joining of independent genes, a variety of fusion products with diverse functional impact can arise. It is important to separate the translocation event on the DNA level from the fusion product that is eventually transcribed on the RNA level. Even though translocations may occur in intronic regions in vicinity of an exon, these events do not always lead to fusion and transcription of adjacent exons. While a translocation can be explicitly described on the DNA level by naming the translocation partners alongside the genomic breakpoints in a specific reference genome, the annotation of the resulting gene fusion on RNA level needs much more consideration. The annotation has to provide vital information to an MTB to assess possible therapeutic decisions. Another example highlights potential pitfalls when applying evidence classifications to translocation events. Evidence for crizotinib in ALK rearranged NSCLC was generated using fluorescence in-situ hybridization, the companion diagnostics assay in the approval trial and gold standard at that time. This assay is not able to differentiate individual fusion partners or provide information beyond a translocation event within the ALK gene. Hence a strict application of the classification systems might assign a high evidence level to a translocation involving the ALK gene locus, even in cases where functional tyrosine kinase domains are missing in the chimeric protein. Consequently, and identical to evaluation of other MBs, precise assessment of a potential biological relevance of a novel fusion is a crucial step following annotation. Additional important considerations include protein interactions, such as dimerization-capability of fusion proteins that leads to ligand-independent activation, determination of subcellular localization or fusion protein stability. Several publicly available databases (e.g., Mitelman Database, COSMIC, ARCHER Quiver, Tumor Fusion Gene Data Portal) and resources combining multiple databases as well as incorporating published data (e.g., ChimerKB) exist to query a detected fusion. While the information contained in these databases increases the plausibility of a novel fusion, it is often not sufficient for further functional evaluation.

6.2 Complex and composite genomic biomarkers

TMB and HRD represent recent examples of complex biomarkers extending the concept of alterations affecting a single or a few genes to alteration patterns that spread over the entire genome.
TMB is used as companion diagnostics for immune checkpoint inhibitor therapy, while the HRD score is used as companion diagnostic for PARP-inhibitors. Implementation of these biomarkers into clinical practice is associated with scientific and technical challenges including:

- accurate definition of the biomarker in a manner that is optimized to answer the underlying clinical question,
- implementation of technology and IT for high-throughput NGS and measurement of the biomarker in routine diagnostics,
- standardization of laboratory and bioinformatic workflows to achieve valid and reliable measurement of the biomarker,
- inter-assay calibration and optimization of cut-off points to translate the measured biomarkers levels into a clinical decision,
- inherent probabilistic nature, particularly when focused assays are being used that do not cover the full genomic footprint of interest.

### 6.2.1 Tumor mutational burden

Originally, TMB was defined as the number of somatic missense mutations in the tumor exome. Depending on the assay and study, not only missense mutations but other types of mutations including indels and nonsense mutations are included, and some assays also consider synonymous variants. Prior to calculation of the TMB, mutations have to be called and appropriately filtered. Filters include a minimum sequencing depth and a minimum VAF which need be met for a variant to be included in the TMB. In a recent pan-cancer study, clonal TMB was a strong predictor of response to immune checkpoint inhibition (ICI), while subclonal TMB failed to reach significant predictivity, supporting a view that mutations with low VAF do not need to be considered for the calculation of TMB.

Once calibration of TMB levels between different platforms is achieved, consistent TMB classification across technical platforms and across laboratories is feasible. In particular, the percentage of strong misclassifications is limited when a 3-tier classification system with a grey zone including tumors with intermediate TMB is used.

**Example**: In a pre-specified exploratory analysis of the KEYNOTE-158 study, a higher likelihood of objective response with pembrolizumab monotherapy was observed in tumors with high TMB (TMB-H, 29%) compared with patients with low TMB (TMB-L, 6%). While discussions on the optimal method to define and measure TMB are ongoing, TMB classification in this study was based on the F1CDx assay (Foundation One) with a cut-off point at 10 mutations/MB. Based on the KEYNOTE-158 study, the FDA approved pembrolizumab for the treatment of patients with resectable or metastatic TMB-H solid tumors, that have progressed following prior treatment and who have no satisfactory alternative treatment options. As a consequence, elevated TMB in small cell lung carcinoma (SCLC) is classified as Tier I-A, I-C, and m1B-Z using JCR, ESCAT, and NCT classification schemes, respectively. KEYNOTE-158 included a total of 76 patients with SCLC whereof 10/34 of TMB-H and only 4/42 of TMB-L patients showed an objective response ($P = 0.037$). This significant result could justify a classification of TMB-H as I-B instead of I-C within the ESCAT framework (Table 2).

### 6.2.2 Homologous recombination deficiency

Defective DNA repair is accompanied by the accumulation of characteristic genomic scars in cancer cells. Telomeric allelic imbalance (TAI), large-scale state transitions (LST), and loss of heterozygosity (LOH) are prevalent in tumors deficient for homologous recombination. These measures can be combined in a summary score HRD $= \text{TAI} + \text{LST} + \text{LOH}$. Originally, HRD scores were developed based on genotyping data collected using single nucleotide polymorphism arrays. Meanwhile, commercial vendors have developed NGS-based assays for measurement of HRD scores. A high correlation of HRD scores derived from WES data and from genotyping data has been demonstrated. Further studies evaluating the comparability of different platforms for routine diagnostics of HRD will be necessary.

**Example**: PAOLA-1 was a phase 3 trial that evaluated olaparib in combination with bevacizumab as first-line maintenance treatment for advanced high-grade serous ovarian cancer. The addition of olaparib to bevacizumab provided a significant survival benefit, which was substantial in patients with HRD-positive tumors. In this study, HRD status was determined by measuring HRD using MyChoice (Myriad Genetics) and dichotomized at the value of 42. Based on this data, first-line maintenance treatment with olaparib in combination with bevacizumab was approved in high-grade serous ovarian, fallopian tube and primary peritoneal carcinoma with positive HRD status by the FDA and by the EMA (Table 2), and is reflected in the uniform evidence classification of JCR Tier I-A, ESCAT I-A, and NCT m1A-Z.

### 7 Integrated evaluation of NGS data by the molecular tumor board

The molecular tumor board is a multidisciplinary panel of (molecular) pathologists, oncologists, clinical geneticists, bioinformaticians, molecular biologists, and specialists from additional clinical disciplines (e.g., radiology, radiation therapy, nuclear medicine). MTBs play a decisive role in the process of variant interpretation. Tasks of the MTB consist of analysis, integration, evaluation, presentation, and discussion of the clinical and molecular data, which results in identification and prioritization of possible therapeutic options based on MBs. This prioritization adheres to the standards of medical decision making and has to consider a multitude of variables beyond the aforementioned MB-drug associations. These are, among others: a patient’s medical history and comorbidities, availability, and adverse effects of recommended treatments, as well as pharmacogenomics and pharmacokinetics. Different MTBs vary in terms of their composition, tasks and work processes, which can lead to variability in the quality of oncological care.

With knowledge of this constellation, local, and national structures strive for increasing standardization and harmonization of
precision oncology and MTBs. These activities require a structured and standardized exchange of information and data to enable harmonized interpretation of genetic data sets. In order to make the best possible use of the accumulated expertise of the MTBs, an optimal processing of the available molecular and clinical information is a condition sine qua non.

MTBs are currently facing additional challenges. First, the analysis of an increasing number of complex (and composite) MBs must be seamlessly integrated in the evaluation of the molecular results from large NGS panels, WES, and WGS in order to identify the biological mechanisms of sensitivity and resistance to specific drugs. Second, clinically relevant thresholds for using novel MB have to be carefully benchmarked. Third, with a growing number of published clinical trials of molecularly targeted (and non-targeted) combination therapies, molecular evidence for combination therapies has to be actively evaluated during MTB deliberation, for example, immune checkpoint blockade and BRAF/MEK inhibition in BRAF V600 mutated melanoma. Moreover, estimation of clinical benefit of rare MB-drug associations based on increasingly smaller cohorts of patients is challenging. Assessment of multiple biomarkers and recommendation of therapeutic drug combinations as well as evaluation of inclusion criteria for molecularly-stratified clinical trials belong to the additional tasks of the MTB.

8 | CONCLUSION

Molecular pathology and precision oncology are rapidly growing interdisciplinary fields of translational and clinical medicine, which drive the development and application of molecularly informed therapy of cancer. With the implementation of NGS in routine care of oncological patients, the standardized evaluation of multiple, individual somatic variants is a task of paramount clinical importance. The clinical value of NGS is based not only on the correct identification, but also on the careful interpretation of oncogenic variants, which can yield clinically valuable diagnostic, prognostic and therapeutic information. The successful implementation of MTBs in clinical routine as well as in observational trial protocols can offer a significant clinical benefit to cancer patients. Heterogeneity and complexity of clinical and molecular datasets increase the effort required for careful interpretation and standardized reporting. The development of an experienced multidisciplinary MTB is therefore one of the most urgent challenges in precision oncology, for which increasing harmonization is necessary. In Germany, a first step toward harmonization was achieved by implementing the NCT classification within several networks of precision oncology.

In this review, we outlined the sequential steps in the variant interpretation process consisting of: (i) quality-assured molecular diagnostics, (ii) standardized variant annotation followed by (iii) functional classification, and (iv) evaluation of clinical significance (Figure 1). We propose a working model for variant curation and interpretation and expose the current challenges in the analysis of somatic variants in MTBs.

One goal of precision oncology and molecular pathology should be the creation of databases and standards in order to quickly identify those therapeutic approaches that add value for patients and to separate them from ineffective or even harmful interventions.

In addition, translational research programs benefit from the harmonization of variant interpretation and lead to the development of innovative translational and clinical research projects as well as clinical trials. Centralized, scientifically valuable databases, programs or registry studies developed in national and international consortia, such as the Centers of Personalized Medicine (ZPM) and the German Network for Precision Medicine (DNPM) in Germany, or the Cancer Core Europe (CCE), provide the “critical mass” necessary for rapid clinical translation of precision medicine and sustainable improvement of patient outcomes.

ACKNOWLEDGMENTS

Open access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Peter Horak https://orcid.org/0000-0003-4536-9306
Michael Altgäuer https://orcid.org/0000-0003-4518-7887
Christian P. Schaaf https://orcid.org/0000-0002-2148-7490
Albrecht Stenzinger https://orcid.org/0000-0003-1001-103X

REFERENCES

1. Westphalen BC, Bokemeyer C, Buttner R, et al. Conceptual framework for precision cancer medicine in Germany: consensus statement of the Deutsche Krebshilfe working group ‘Molecular diagnostics and Therapy’. Eur J Cancer. 2020;135:1-7.
2. Luchini C, Lawlor RT, Milella M, Scarpa A. Molecular tumor boards in clinical practice. Trends Cancer. 2020;6(9):738-744.
3. Horak P, Frohling S, Glimm H. Integrating next-generation sequencing into clinical oncology: strategies, promises and pitfalls. ESOM Open. 2016;1(5):e000094.
4. Wahjudi LW, Bernhardt S, Abnaof K, et al. Integrating proteomics into precision oncology. Int J Cancer. 2021;148(6):1438-1451.
5. Kato S, Kim KH, Lim HJ, et al. Real-world data from a molecular tumor board demonstrates improved outcomes with a precision N-of-one strategy. Nat Commun. 2020;11(1):4965.
6. van der Velden DL, Hoes LR, van der Wijngaart H, et al. The drug rediscovery protocol facilitates the expanded use of existing anticancer drugs. Nature. 2019;574:127-131.
7. Jardim DL, Schweanderle M, Wei C, et al. Impact of a biomarker-based strategy on oncology drug development: a meta-analysis of clinical trials leading to FDA approval. J Natl Cancer Inst. 2015;107(11):dvj253.
8. Massard C, Michiels S, Ferté C, et al. High-throughput genomics and clinical outcome in hard-to-treat advanced cancers: results of the MOSCATO 01 trial. Cancer Discov. 2017;7(6):586-595.
9. Rodon J, Soria JC, Berger R, et al. Genomic and transcriptomic profiling expands precision cancer medicine: the WINThER trial. Nat Med. 2019;25(5):751-758.
10. Sicklick JK, Kato S, Okamura R, et al. Molecular profiling of cancer patients enables personalized combination therapy: the I-PREDICT study. Nat Med. 2019;25(5):744-750.
52. Stenzinger A, Allen JD, Maas J, et al. Tumor mutational burden standardization initiatives: recommendations for consistent tumor mutational burden assessment in clinical samples to guide immunotherapy treatment decisions. Genes Chromosomes Cancer. 2019;58(8):578-588.

53. Stenzinger A, Endris V, Budczies J, et al. Harmonization and standardization of panel-based tumor mutational burden measurement: real-world results and recommendations of the quality in pathology study. J Thorac Oncol. 2020;15(7):1177-1189.

54. Budczies J, Allgauer M, Litchfield K, et al. Optimizing panel-based tumor mutational burden (TMB) measurement. Ann Oncol. 2019;30(9):1496-1506.

55. Abkevich V, Timms KM, Hennessy BT, et al. Patterns of genomic loss of heterozygosity predict homologous recombination repair defects in epithelial ovarian cancer. Br J Cancer. 2012;107(10):1776-1782.

56. Birkbak NJ, Wang ZC, Kim JY, et al. Telomeric allelic imbalance indicates defective DNA repair and sensitivity to DNA-damaging agents. Cancer Discov. 2012;2(4):366-375.

57. Popova T, Manie E, Rieunier G, et al. Ploidy and large-scale genomic instability consistently identify basal-like breast carcinomas with BRCA1/2 inactivation. Cancer Res. 2012;72(21):5454-5462.

58. Sztupinszki Z, Diossy M, Krzystanek M, et al. Migrating the SNP array-based homologous recombination deficiency measures to next generation sequencing data of breast cancer. NPJ Breast Cancer. 2018;4:16.

59. Gonzalez D, Stenzinger A. Homologous recombination repair deficiency (HRD): from biology to clinical exploitation. Genes Chromosomes Cancer. 2021;60(5):299-302.

60. Ray-Coquard I, Pautier P, Pignata S, et al. Olaparib plus bevacizumab as first-line maintenance in ovarian cancer. N Engl J Med. 2019;381(25):2416-2428.

61. Gutzmer R, Stroyakovskiy D, Gogas H, et al. Atezolizumab, vemurafenib, and cobimetinib as first-line treatment for unresectable advanced BRAF(V600) mutation-positive melanoma (IMspire150): primary analysis of the randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2020;395:1835-1844.

62. Dickson D, Johnson J, Bergan R, Owens R, Subbiah V, Kurzrock R. The master observational trial: a new class of master protocol to advance precision medicine. Cell. 2020;180(1):9-14.

63. de Vries EGE, Cherny NI, Voest EE. When is off-label off-road? Ann Oncol. 2019;30(10):1536-1538.

64. Tamborero D, Dienstmann R, Rachid MH, et al. Support systems to guide clinical decision-making in precision oncology: the Cancer Core Europe Molecular Tumor Board Portal. Nat Med. 2020;26(7):992-994.

How to cite this article: Horak P, Leichsenring J, Goldschmid H, et al. Assigning evidence to actionability: An introduction to variant interpretation in precision cancer medicine. Genes Chromosomes Cancer. 2022;61(6):303-313. https://doi.org/10.1002/gcc.22987