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Molecular pathology of cancer: how to communicate with disease

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ABSTRACT

Recent technical advances have brought insights into the biology of cancer in human, establishing it as a disease driven by genetic mutations. Beside inherited family tumour syndromes caused by germline mutations, somatic genetic alterations occur early in tumourigenesis, which accumulate during the progression of the disease and its treatment. Based on these observations, medical oncology has started to enter an era of stratified medicine, where treatment selection is becoming tailored to drugable molecular pathways. As a pre-requisite of an individualised treatment concept, molecular and genetic characterisation of the individual tumour has to be performed to align the most appropriate therapies according to the patient's disease. Reading the individual molecular tumour profile and responding by a tailored treatment concept is the 'communication' required to fight this deadly disease. This way to communicate is currently changing the field of oncology dramatically, and fundamentally involves the discipline of molecular pathology. This review highlights the role of genetic characterisation of human malignancies by giving an overview on the basic methods of molecular pathology, the challenge of the instable tumour genome and its clinical consequences.

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THE ROLE OF PATHOLOGY IN CANCER DIAGNOSTICS—FROM THE DISSECTION ROOM TO MOLECULAR PATHOLOGY

To understand the role of molecular pathology in today's oncology, it is necessary to obtain a historical view; when—in the absence of other treatment options—the therapy of patients with cancer was limited to surgery (if possible). In this setting, the relevance of oncological pathology was also very limited.

In the early 19th century, pathology in tumour diagnostics was more or less restricted to a postmortem description of macroscopic tumour spread in the dissection room. But things changed dramatically with the establishment of light microscopy in the second half of the 19th century by Rudolf Virchow (1821–1902) who is considered the 'father of modern pathology'.[1] Now it became possible to investigate the microscopic structure of tumours, allowing pathologists to create new classifications of malignant diseases. The next big step forward was the pioneering work in the field of (gynaecological) cytology by George N Papanicolaou in 1920.[2]

This method allowed detecting even precursor lesions of cervical cancer at a time when they were still easily curable. Also, owing to the development of various biopsy technologies, pathological findings became increasingly relevant for the clinical management of patients with cancer: it was now possible to perform a biopsy before surgical removal of a suspected lesion, thus avoiding unnecessary procedures in benign conditions or very advanced diseases. Oncological research also demonstrated that the prognosis for patients and adequate clinical management of patients with cancer was associated with histological findings: morphologically distinct tumour entities often behave differently, and tumour spread, for example, to lymph nodes or to resection margins that are detectable only microscopically might significantly influence the outcome of patients. With the development of radio-oncology in the first half and the rise of modern chemotherapy in the second half of the 20th century, it also became evident that distinct morphological tumour entities at the same location might respond completely different to radiotherapy and/or chemotherapy. These findings were to change pathology fundamentally: instead of delivering only postmortem descriptions of cancer spread without any benefit to the (already deceased) patients, clinical decisions became more and more frequently based on pathology findings.

The rise of 'precision medicine' began in the 1980s with the development of immunohistochemistry.[3] This method permitted pathologists to investigate relatively easily the expression of various proteins on histological slides obtained from surgical specimens. These expression levels would soon turn out to be relevant for subclassifications of tumours that were not accessible by light microscopy alone.

But the situation was to change dramatically when therapies became available which targeted special proteins detectable by immunohistochemistry: if the respective proteins were expressed, these therapies should be administered, but if no expression was evident, such therapies were not indicated.

The first 'targeted therapy' to attain relevance in a common human cancer was the blockade of oestrogen receptor signalling in patients with breast cancer suffering from tumours that showed overexpression of this protein.[4 5]

The next strategic landmark of individualised oncology was the establishment of the blockade of overexpressed HER2/neu antigen also in patients with breast cancer.[6]

In spite of the detection of relevant changes in protein expression levels, which are mainly caused by genetic alterations (eg, the amplification of the HER2 gene), investigations at the genetic level in particular have moved into the focus of interest in pathology. While until recently, the sequencing or detection of structural alterations of single genes was considered state-of-the-art, the rise of next generation sequencing has changed the scenario dramatically.[7 8] Using these methods, a large number of genes can be investigated by just one single investigation to deliver an enormous amount of information. This 'information overflow' represents a major challenge in modern pathology, since —in spite of the bioinformatics challenge—a vast number of genetic alterations are detected, the clinical relevance of which is very often not clear.

It is therefore crucial for molecular pathology to find the balance between technical, potential and clinical usefulness of investigations.

Thus, at the moment, pathology is again undergoing dramatic changes, comparable to the shift from the dissection room to light microscopy in the 19th century.

Whereas the analysis of immunohistochemistry is equally morphology-based, thus not representing a real breakthrough in the historical tradition of light microscopy since the days of Virchow, modern pathology is getting increasingly different: classical morphology is subsequently enhanced by novel molecular technologies in which 'classical' morphology is no longer of relevance. The modern pathologist needs to ascribe increasing importance to detailed knowledge in morphology and in molecular mechanisms of cancer.

MOLECULAR MECHANISMS IN CANCER

The fact that cancer is a genetic disorder was suspected amazingly early in the history of cancer research: as early as in 1914, Theodor Boveri presented a systematic somatic mutation theory of cancer. According to Boveri's hypothesis chromosomal changes caused the transition from normal to malignant proliferation.[9]

It took almost 50 years to provide conclusive verification of Boveri's idea, when Nowell and Hungerford[10] found a small karytopic marker, the Philadelphia chromosome, in patients with chronic myeloid leukaemia (CML). After the discovery of the Philadelphia chromosome in 1960, it took another 10 years to demonstrate that the Philadelphia chromosome resulted from a reciprocal chromosome translocation involving chromosomes 22 and 9, and yet another 10 years to map the breakpoints to the Abelson murine leukaemia viral oncogene (ABL) and breakpoint cluster region genes.[11 12] This was to change the scenario fundamentally. The sequence of basic biological discoveries culminated in successful therapeutic targeting of CML and Ph-positive acute lymphoblastic leukaemia via small-molecule ABL kinase inhibitors. CML thus became the paradigm for targeted treatment in cancer, directed by the underlying genetic abnormality.

The technology-driven increase of knowledge in cancer biology is no longer restricted to tumour genetics of a visible tumour, but affects many areas such as stem cells, microenvironment or clonal evolution. Furthermore, clinicians are not faced with a genetic tumour report of a tumour biopsy alone, but will depend on reports for minimal residual disease, clonal evolution or liquid biopsy. In contrast to the long period of time required for verification of CML as outlined above, we nowadays have the potential to sequence the cancer genome of each patient at an affordable price within a short period of time. In this review, we will focus on advances in understanding the molecular pathology of cancer with respect to clinical usability.

CANCER PREDISPOSITION

Although the majority of cancer cases may represent occasional events, healthcare providers should be encouraged to consider a hereditary condition. Breast/ovarian cancer or colon cancer are prominent examples for hereditary cancer syndromes, but represent only a small fraction of known hereditary cancer predisposition syndromes.

The American College of Medical Genetics and Genomics (ACMG) developed referral guidelines for 28 of the most common hereditary cancer susceptibility syndromes, enabling clinicians to quickly search by cancer type.[13] Suspicion of a hereditary cancer syndrome may result in genetic testing of the index case. Many models have been developed to estimate the likelihood that an individual or family has a germline pathogenic variant, such as *BRCA1* or *BRCA2*.[14] The list of genes related to hereditary cancer syndromes has grown over time and gone beyond the testing of single genes. In breast/ovarian cancer, mutation testing is not restricted to *BRCA1* and *BRCA2* anymore, but is complemented by further loci. Genes, such as *PALB2*, *TP53* (associated with Li-Fraumeni syndrome), *PTEN* (associated with Cowden syndrome), *CDH1* (associated with diffuse gastric and lobular breast cancer syndrome) and *STK11* (associated with Peutz-Jeghers syndrome), confer a risk

to either or both of these cancers with relatively high penetrance. Additional genes, such as *CHEK2*, *BRIP1*, *RAD51* and *ATM*, are associated with breast and/or gynaecological cancers with moderate penetrance. Once a germline mutation is established in the index case, reliable testing of further family members is possible. Hereditary cancer syndromes are usually inherited in an autosomal dominant manner with a 50% chance of inheriting the variant in the offspring of an individual germline pathogenic variant. The detection of a pathogenic variant does not necessarily lead to a tumour because of incomplete penetrance, variable age of cancer development, cancer risk reduction resulting from prophylactic surgery or early death. The risk of developing a cancer typically increases over time and is not restricted to one cancer type, but may involve different tissues with varying likelihood. In summary, management of families with hereditary cancer is a complex issue involving many disciplines.

SOMATIC MUTATIONS

Irrespective of a potential underlying genetic predisposition, there is increasing evidence that cancer reflects a multistep evolutionary process. In 1990, Fearon and Vogelstein[15] developed a multistep model for the development of colorectal cancers. In their proposed model of successive steps, they included mutations in the activation of oncogenes, such as KRAS, and inactivation of both alleles of tumour suppressor genes, such as TP53, exemplifying Knudson's two-hit hypothesis. Activation or inactivation of oncogenes and tumour suppressor genes, respectively, may be the result of different types of mutations including chromosomal rearrangements or gene mutations. Many of these changes are non-random and even sometimes mandatory, such as *MYC* translocation in Burkitt lymphoma. Nowadays, the search for cancer drivers is no longer restricted to the classical model of oncogenes and tumour suppressor genes. Many novel mechanisms have been detected to be involved in tumour pathogenesis. For example, the inclusion of deregulated cellular energetics as a hallmark of cancer reflects the increasing recognition of this fundamental cellular process in malignant transformation. The first mutations discovered in genes encoding isocitrate dehydrogenases (IDHs; including IDH1 and IDH2) were identified in metastatic colon cancer and this discovery represents one of the highlights of cancer biology research in the era of high-throughput sequencing.[16] IDH enzymes have become a focal point for research aimed at understanding the biology of glioma.[17] New sequence technologies allow identifying virtually all somatic changes, but clearly the majority of them have no clear consequences and a tiny minority foster progression. It has been shown that different mutational processes generate different combinations of mutation signatures.[18–20] Certain signatures are associated with the age of the patient at cancer diagnosis, known mutagenic exposures or defects in DNA maintenance, but many are of cryptic origin.[21]

UNDERSTANDING OF NOVEL TECHNOLOGIES

The understanding of the different technologies now increasingly used in pathology is nowadays an important piece of information for clinical management. The WHO Classification of Tumours of the Haematopoietic and Lymphoid Tissues published in 2001 reflected a paradigm shift in the approach to classification of myeloid neoplasms.[22] For the first time, genetic information was incorporated into diagnostic algorithms provided for the various entities. At the beginning, predominantly chromosomal abnormalities were included in evidence-based classifications and intended to be used in daily practice for therapeutic decisions. Techniques for the detection of chromosomal abnormalities vary. In an ideal situation, fresh tumour material is cultured, tumour cells grow in cell culture and metaphases of the dividing tumour cells are obtained for cytogenetic analysis. In this situation, virtually all cytogenetic abnormalities are visible and can be reported. However, tumour growth may be challenging in cell culture, or the available material is too small. The major drawback is the fact that the vast majority of tumour samples intended for genetic testing is available as paraffin-embedded tissue only.

This formalin fixation, paraffin embedding (FFPE) is in general a major problem for molecular technologies, but this fact reflects the routine workup of specimens.

This problem cannot be bypassed easily in tissue-based molecular pathology of cancer, because it is relevant to know what kind of tissue is subjected to further (molecular) investigations, and a precise 'traditional' morphological assessment of each sample should be mandatory. 'Some' result will be obtained, for example, by sequencing in most cases, but without a preceding histological evaluation of the sample to be analysed it is not certain how much (if any!) tumour tissue is being investigated. Therefore, all novel techniques for the investigation of tissue samples must account for the fact that the tumour material is distinctly altered by fixation and embedding.

In this FFPE setting, fluorescence in situ hybridisation (FISH) proved a powerful tool to detect gains or losses of genetic material such as HER-2/neu amplification in breast cancer.[23] The unique feature of FISH is the ability to detect chromosomal translocations of one gene irrespective of the fusion partner. FISH probes can be designed such that two differently labelled probes flank the complete breakpoint region of the gene of interest and separate signals are the proof of the translocation. The detection of anaplastic lymphoma kinase translocations in non-small cell lung cancer (NSCLC) tissue specimens is a typical example of FISH translocation testing.[24] Apart from chromosomal abnormalities, mutation testing of the coding regions of genes is the major area of genetic testing. The online Catalogue of Somatic

Mutations in Cancer (COSMIC) is a valuable source for known activating and inactivating genes in cancer genes. Inactivating mutations such as non-sense mutations, splice site mutations, and frame-shift deletions and insertions are easy to interpret. Missense mutations with the exchange of an amino acid by another amino acid may be more difficult to interpret, especially when information from the literature is missing. Sequence analysis was performed by classical Sanger sequencing for a long period of time.[25]

This robust method has the disadvantage that the analysis of large genomic regions is too expensive and that the sensitivity for mosaic mutations is rather low. The implementation of high-throughput sequencing techniques overcomes some of these drawbacks. Typically, gene panels or the entire coding region of all genes (exome) are enriched and sequenced with multiple coverage. The increase of coverage leads to higher sensitivity and detection of small tumour clones on the one hand and higher costs on the other hand. Owing to the possibility of performing ultradeep sequencing, new opportunities arise in cancer diagnostics. One of the most recent, exciting areas of tumour genetics is the sequence analysis of cell-free tumour DNA isolated from plasma or urine (commonly called liquid biopsy). Many laboratories start nowadays with the detection/monitoring of known hot spot mutations (relevant for therapy resistance) in EGFR, KRAS or BRAF, but exome or genome sequencing is in fact technically possible from cell-free tumour DNA. These new tests have the major advantage that circulating cell-free tumour DNA represents the tumour activity of many (visible and undetected) tumour localisations of a patient and is not restricted to the analysis of one biopsy. Furthermore, blood samples can be obtained more easily and more frequently compared with surgical procedures. The sensitive methods have also demonstrated that mutations that were believed to appear during disease progressions are already present at early tumour stages.[26] These findings suggest that resistant clones are present before treatment, which would make up-front therapeutic combinations that target non-overlapping resistance a preferred approach.

Another novel technology which is increasingly used for therapy decision-making, especially in breast cancer, is the analysis of gene expression signatures. Various assays using different technological platforms are available, but they all have in common that the expression of a subset of defined genes is analysed in FFPE of the tumours, and cancer-specific risk scores are calculated.[27] These scores predict the risk of recurrence and should therefore help clinicians with therapy selection.

Another highly promising future technology in pathology might be mass spectroscopy.[28]

Here, the expression of hundreds of proteins may be investigated simultaneously, which is also possible by a morphology-based approach using slides from formalin-fixed, paraffin-embedded tumour specimens. This technology may have the potential to replace immunohistochemistry in a variety of applications: when, for sufficient diagnosis of a tumour, a variety of subsequent single immunohistochemical investigations is required, mass spectroscopy might deliver the required results cheaper and faster with one single investigation, and might (if slide-based) also give clear information concerning co-expression of proteins. Until now, this technology is still under development and widespread use in diagnostic pathology is not foreseeable in the near future.

Nevertheless, several central laboratories are already offering multiplex targeted proteomic analyses in a certified environment and first data show that for clinical implications, this approach seems at least comparable, if not superior, to standard immunohistochemistry.[29 30] One of the major points will be, whether sensitivity and resolution of morphology-based mass spectroscopy can be increased.

In summary, the development of novel technologies has dramatically increased the ability to obtain information on molecular alterations from cancer samples.

The main question that remains is into which clinical benefit for patients with cancer these findings might be translated.

CLINICAL IMPACT AND PERSPECTIVES

Although there is a general acceptance towards an individualised treatment approach to stratify and subgroup patients with the aim to improve the quality of clinical care in oncology, molecular profiling has just started to assist the prediction of the drug's clinical benefit by identifying the most responsive patient subgroup.

Recently, excellent demonstrations of the utility of prognostic and/or predictive biomarkers have emerged. Von Hoff *et al*[31] have recently demonstrated that molecular profiling of patients' tumours is an efficient approach to identify potential targets and select treatments for their treatment-refractory cancers. Such a tailored treatment strategy revealed to be an effective approach to increase progression-free survival (PFS), when compared with the patients' most recent standard treatment regimen. Another example for individualised treatment of patients is given by the recently published BATTLE trial (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination), a prospective biomarker and biopsy-driven trial in pretreated patient with NSCLC.[32] By this Bayesian approach, the authors demonstrated that targeting individually analysed molecules of the patient's tumour might represent an efficient therapeutic approach in the treatment of an incurable disease.

While these examples suggest a benefit for individual tumour characterisation for the selection of a tailored treatment concept, this concept did not meet the primary study aim in the SHIVA01 and the SAFIR trial. One weakness of the SAFIR trial was the fact that the molecular profiles of the respective tumours led to a

predefined subgrouping of patients resulting in respecting treatment concepts.[33] This experimental stratified treatment concept failed to improve PFS compared with the control arm. In the SHIVA01 trial, the primary end point was to include at least 30% of patients in clinical trials for testing a targeted therapy, but only in 13% of patients with breast cancer, a drugable target was detected.[34] Similar to the SAFIR trial and in contrast to Van Hoff's pilot trial, the panel of molecular mutations tested was limited.

In 2013, we initiated the clinical trial 'EXACT' focusing on a personalised treatment approach in patients with solid tumour after the failure of standard treatment options (EK1541/2012, https://ekmeduniwien.at/core/catalog/2012/). In an interim analysis performed after 30 patients had been treated with an individualised treatment approach according to the real-time molecular profile of the respective tumour tissue section, we learnt that especially patients with gastrointestinal tumours seem to benefit from this treatment strategy. Specifically, 13 out of 30 patients (43%) had a tumour derived from the gastrointestinal tract. Of these, 10 (77%) patients had a clinical benefit when compared with their previous treatment, which was the predefined primary study end point. We observed long-term treatment benefits on experimental treatment according to the patient's molecular tumour profile, remarkably in favour of the individual overall survival expectancy. Notably, none of the 30 patients developed severe side effects (≥grade 3 side effects) in this heavily pretreated population of patients with non-curable cancer.

In addition to our findings, recent evidence suggests that individualised treatment approaches might be beneficial for patients suffering from non-resectable gastrointestinal cancer. Thus, the HERACLES trial suggested that in patients with Her2+ overexpressing colorectal cancer (metastatic colorectal cancer, mCRC) a combination treatment with lapatinib plus trastuzumab might be beneficial after the failure of anti-EGFR treatment.[35] The authors found that in 74% of these heavily pretreated patients, a disease control could be achieved. Although Her2+ overexpression in mCRC is rare (2–10%) the absolute number of patients who might be eligible for such treatment is comparable to mutated NSCLC, gastrointestinal stromal tumour or CML—diseases in which targeted therapies are in routine clinical use. In mismatch-repair deficient gastrointestinal cancer, anti-PD-1 antibody treatment was recently suggested to have a major benefit for patients (HR for disease progression or death, 0.10 ($p<0.001$), and HR for death, 0.22 ($p=0.05$)). Thus, 10–15% of patients with mCRC might benefit from immunotherapy.[36]

CLINICAL OUTLOOK

In summary, currently novel targeted agents in the treatment of cancer are approved for a certain subtype of cancer rather than for patients based on the expression or activity of respective target lesions. The need of an extension of clinical protocols focusing on molecular profile-based treatment decisions, rather than on anatomic cancer subtypes is mandatory.

Generally, the importance of molecular pathology in oncology will increase in the very near future, since molecular diagnostics will not represent just a single investigation at the beginning of patient management any more: especially liquid biopsies will establish molecular pathology as a continuous monitoring regimen during the course of disease. This technique allows the detection of cancer recurrences earlier than radiology, thus allowing to adapt therapies earlier.[37] In addition, the development of gene mutations associated with resistance to administered therapies might also be detected very early on.[38]

Thus, the fields of pathology and oncology will be subjected to even more fundamental changes than those already seen, most probably realising the 'dream of precision medicine' in the years to come.

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